# INTERACTION OF COPPER AND ORGANOTIN WITH MARINE BACTERIA

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To my mother

and

in memory of my father

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ABSTRACT

A range of marine bacteria was isolated from the natural marine environment and characterized with respect to morphological, physiological and biochemical features. Surfaces coated with antifouling paints were found to select for a specific range of bacteria.

The effect of copper and organotin on growth and morphology of a number of these isolates was determined. The differing abilities of isolates to tolerate toxicants suggested that, within the diverse marine microbial community, there will exist bacterial species tolerant of antifouling paints. Studies on the interaction of toxicants with marine bacteria were performed mainly using isolates 82Q and S3/A51, which showed relatively low and high levels of resistance respectively.

Morphological changes observed during growth in the presence of copper suggested that cell wall synthesis and cell division were targets for toxic action. Biosynthetic pathways were also indicated as sites of toxic action, since changes were observed in the nature of extracellular polysaccharides and outer membrane proteins produced during growth in the presence of copper or organotin.

Determination of the influence of toxicants on bacterial attachment to surfaces revealed that attachment of isolate 82Q was greatly reduced in the presence of copper or organotin. This effect was accompanied by a loss of cell viability. Isolate S3/A51 showed less reduction in attachment, with no concomitant loss of viability.

This suggested that attachment of some cells was prevented by interaction of toxicants with cell surface components involved in the attachment process. Copper had a similar effect on the attachment of a copper resistant mutant 82Q/15b, when it had previously been grown in the presence of copper.

Comparison of extracellular polysaccharides from these isolates revealed that, although they contained similar monosaccharide constituents, these were present in different molar ratios. This might result in distinct polysaccharide structures and thus determine the attachment properties of the two isolates. The greater tendency of polymer from 82Q to interact with cations may also be implicated in reduction of attachment.

It was proposed that tolerance to toxicants is inducible, and may be dependent on control of their uptake by the cell. The strategies adopted by different species to effect this control, and their relative successes, were discussed. Extracellular polysaccharides and/or outer membrane proteins were implicated in tolerance mechanisms. S3/A51 responded to toxicants by production of a polysaccharide with a possible altered structure, and altered minor outer membrane proteins. Immobilized metal affinity chromatography revealed that one such protein, repressed in the presence of copper, bound strongly to copper. Since this isolate showed greater tolerance, these strategies appeared to be more successful in controlling uptake of toxicants and thus conferring tolerant properties on the cell.

DECLARATION

I hereby declare that this thesis has been composed by myself, and that the work presented herein is my own. Any collaborative work has been duly acknowledged.

LORNA MILLER

1985

## ABBREVIATIONS

| | |
|---|---|
| ASW | Artificial seawater |
| ATP | Adenosine triphosphate |
| Dex. | Dextrose |
| DMTC | Dimethyltin dichloride |
| DNA | Deoxyribonucleic acid |
| Dul. | Dulcitol |
| EDTA | Ethylenediaminetetraacetic acid |
| Fru. | Fructose |
| Fuc. | Fucose |
| HPLC | High performance liquid chromatography |
| KDO | 2-keto-3-deoxyoctonic acid |
| Lac. | Lactose |
| LPS | Lipopolysaccharide |
| Mal. | Maltose |
| Man. | Mannose |
| Manl. | Mannitol |
| NADH | Reduced nicotinamide adenine dinucleotide |
| Rha. | Rhamnose |
| Suc. | Sucrose |

CONTENTS

CHAPTER TWO: MATERIALS & METHODS

# CHAPTER ONE: INTRODUCTION

## 1.1 General introduction.

For many years it has been apparent to man that virtually every surface submerged in the sea, or in freshwaters,eventually becomes colonized by a wide variety of microorganisms. This constitutes the first step in a well documented succession in the development of a marine fouling community. The formation of such primary films favours the subsequent attachment of larger fouling organisms (Zobell & Allen, 1935) by forming a mucilaginous surface to which they can adhere, and by acting as a food source for them (Young & Mitchell, 1973). Early reports by such workers as Zobell & Allen (1935) and Heukelekian & Heller (1940) first stimulated an interest in the relationship between bacteria and solid surfaces. However, it is only in recent years that the significance of microbial attachment and its associated slime layers has been recognized and methods have been developed to focus on the problem.

Attached bacterial growths in aquatic systems can occasionally be used to man's advantage. Some examples of beneficial effects are described by Characklis & Cooksey (1983), and include trickling filters and rotating biological contactors for wastewater treatment processes, and immobilized organisms for conducting specific chemical transformations in the chemical process industry. More often, microbial biofilm formation results in problems, such as fouling of heat exchange surfaces. (Bott et al., 1983), increased frictional resistance in water-carrying pipelines, and marine fouling of submerged surfaces.

Marine fouling invariably interferes with the efficient operation of structures and, particularly for shipping, is a major economic consideration. The frictional resistance of the vessel increases leading to a rise in fuel consumption, loss of speed and additional stress on the engine. There are also potential problems in the oil industry. Microbial attachment and growth on marine surfaces leads to biodeterioration of the surfaces themselves. The corrosion of metals occurs by essentially electrochemical reactions, but the natures and the rates of these reactions can be influenced by microorganisms. Bacterial fouling produces ideal conditions for metal corrosion by reducing the Eh at the colonized surface to very anaerobic levels. The proliferation of anaerobic biofilms can generate ferrous sulphide particles which are extremely corrosive to mild steel pipes.

Control of marine fouling is fairly limited. For seawater pipes and cooling systems of industrial plants, power stations and ships, chlorine and its derivatives have been effectively used to prevent the settlement stages of fouling organisms. To deter, or retard, the rate of fouling on surfaces exposed to the sea, a range of antifouling paints has been developed. These contain toxic compounds such as cuprous oxide, mercury, arsenic and organotins, which are released into the layer of seawater adjacent to the hull in sufficient concentrations to kill microorganisms or at least to prevent their attachment. Such measures have their limitations, and frequent repainting is necessary as toxin-leaching rate falls below the required level. In addition, the commonly used antifouling paints contain heavy metals which are very toxic and give cause for concern

as pollutants in aquatic environments. Furthermore, the use of these paints has led to the development of fouling microorganisms which are resistant to antifouling agents (Dempsey, 1981a,b). Although only a limited number of bacteria have developed tolerance to toxic paints, development of fouling layers by these bacteria and their secreted mucilage reduces the efficiency of the paints.

These drawbacks underline the need for an evaluation of antifouling paints, and the development of practical, economical methods to control marine fouling. There is a possibility that the development of such methods might arise from a better understanding of the mechanisms involved in and the parameters which affect microbial attachment to surfaces.

Studies on marine antifouling paints have so far revealed little information on the mechanisms of action of the toxicants on fouling bacteria, or on the molecular basis of tolerance to such toxicants. An insight into these processes may also aid in the improvement of measures to limit marine fouling.

## 1.2 Advantages of Attachment for Microorganisms

The ubiquitous nature of microbial biofilms indicates that attachment to surfaces must be advantageous to microorganisms. In some cases, the attachment process and related microbial activities seem to be of fundamental importance to the individual microorganism if it is to survive, replicate and colonize throughout the environment. Dawson *et al.* (1981) suggest that the adhesive properties of starvation-induced dwarf forms of a marine vibrio

enhance their chance of survival in low nutrient waters. However, the possible advantages of attachment in aquatic environments are extremely complex.

Attached organisms appear to have two quite clear benefits in flowing waters. Firstly, fluid flowing over the surface brings fresh nutrients and removes waste products at the same time, thus giving advantage over free-living, motile organisms which must expend energy in order to move into nutritionally favourable environments. Secondly, anchorage to a surface prevents large numbers of the population from being carried by currents to unfavourable environments.

From the very earliest studies of surface-associated growth, it has been postulated that in environments of low nutrient content, such as the sea, the association of bacteria with surfaces either enhances, or is required for their growth, because nutrients become concentrated by sorption to such surfaces. Zobell (1943) showed that demonstrable quantities of organic nutrients were adsorbed from seawater by glass, and that in dilute nutrient solutions, such as seawater, such adsorbed organic material enhanced bacterial activity. Heukelekian & Heller (1940) found that at low nutrient concentrations (< 0.5mg $l^{-1}$ peptone) E. coli could not grow without the addition of glass beads which could provide a larger surface area and a weakly ionic surface encouraging adsorption of nutrients and attachment of bacteria. In these early experiments, it was not clear whether attachment to the added surfaces was a prerequisite for enhanced activity or whether the association was superficial. More recent studies have shown that attached bacteria may exhibit an

elevated growth or metabolic rate compared with that of equivalent suspended populations. Hendricks (1974) demonstrated this with heterotrophic bacteria in river water on the basis of an elevated respiration rate and increased alkaline phosphatase activity for attached bacteria. Ellwood et al. (1982) have shown that the growth rate of a pseudomonad on glass surfaces suspended in chemostat cultures may be several times higher than that of the same organism in the free surrounding medium. Studies performed by Kjelleberg & Dahlback (1984) indicated a higher content of ATP per biovolume (number of cells x cell volume) at solid-liquid interfaces compared with that in the bulk phase. Another possible advantage suggested by Zobell (1943) is that extracellular enzyme activity may be enhanced by attachment. Solid surfaces may retard the diffusion of exoenzymes and hydrolysates away from the cell, thereby promoting the assimilation of nutrients which must be hydrolyzed extracellularly prior to ingestion.

Factors other than nutrient availability and enzyme activity may be involved. Surfaces might provide microenvironments which are more suitable for growth; they may have a more favourable pH than the surrounding environment (Stotzky, 1966a,b); or may provide some protection from phage attack (Roper & Marshall, 1974) by the presence of extracellular polysaccharide (Wilkinson, 1958). The bacteria-surface interactions involved in attachment may have some effect on membrane structure and physiology, thus affecting membrane-associated enzyme activity. Baltzis & Fredrickson (1983) suggest that, in predator-prey studies, bacteria which exhibit attachment to surfaces may be immune to predation.

In contrast, it could be argued that immobilization of cells at a surface may render them more liable to predation.

Another important aspect is the complexity of the bacterial community present in aquatic environments. In mixed bacterial cultures, attachment by one or more bacterial types may affect their competitive ability and thus their relative numbers in the system (Maigetter & Pfister, 1975).

Finally, a recent theory proposed by Ellwood et al. (1982) is based on the chemiosmotic hypothesis of energy conservation, and may account for the increased rate of growth and metabolism seen in surface-associated films. Protons translocated out of the cell during respiration or expenditure of ATP generate an electrical potential across the cell membrane which can be used to drive, in particular, substrate uptake and excretion of metabolic by-products against a concentration gradient. However, as ATP can be generated by the inward flow of protons across the cell membrane, it would be to the cell´s advantage for translocated protons to be retained near the membrane surface. In free-living cells, protons would be likely to diffuse away, but in the case of microbial films, diffusion would be inhibited and cells in a microcolony would benefit from the resultant abundance of available protons.

It should be realized in discussing the ecological advantages of attachment that there is a wide range in the types of communities found on solid surfaces in aquatic environments, thus making it difficult to ascribe any ecological advantages to attached communities in general. The possible advantages will depend on individual conditions such as nutrient concentration, community

diversity and surface characteristics.

## 1.3 Charactistics of Solid Surfaces

In microbial attachment to surfaces, the characteristics of the surface involved will have a major influence. Marszalek et al. (1979) found that surface characteristics influenced microfouling at all stages of development, especially early stages of surface conditioning and primary film formation. In particular, the physicochemical and physical properties, and the tendency for formation of surface conditioning films are of importance.

### 1.3.1 Physicochemical Properties

#### 1.3.1.1 Surface Charge

The surface charge on solid-liquid interfaces may result from the ionisation of surface groups, a phenomenon which is pH dependent. However, the exact nature of these charges and their role in attachment and growth of bacteria is not fully understood. The electrical double layer present at solid-liquid interfaces is well known, and is viewed as a double layer of charged ions. This double layer consists of a fixed layer of charged ions in solution that are firmly bound to the surface, and an outer diffuse layer of less strongly bound ions. The resultant net charge of the two layers balances, and it is the fixed layer which determines surface

charge (fig.1.1). The properties of the electrical double layer are considered by Marshall et al. (1971a) and Marshall (1972). At low electrolyte concentrations, such as in river or estuarine water, the layer is relatively thick. At higher concentrations, such as those found in seawater, it is considerably thinner, and this will have an influence on microbial attachment to the surface (see Section 1.5.2).

It is often found that the surfaces of both the solid and the microorganism are negatively charged and this would presumably lead to mutual repulsion. Fletcher & Loeb (1979) studied the attachment of a marine pseudomonad to a variety of surfaces and found that the number of bacteria which became attached was related to the surface charge. Many bacteria attached to plastics with little or no surface charge, and to metals with a net positive (platinum) or neutral (germanium) charge. However, few bacteria become attached to negatively charged surfaces such as glass, demonstrating the involvement of electrostatic interactions and the importance of surface charge on attachment.

#### 1.3.1.2 Surface Free Energy

The free energy of a surface is defined as the available energy resulting from surface groups, molecules or atoms which are able to interact with other groups, molecules or atoms which may approach the surface; and is an indication of the tendency for the surface to enter into various interactions, such as van der Waals, electrostatic and polar interactions, and chemical bonding (Fletcher

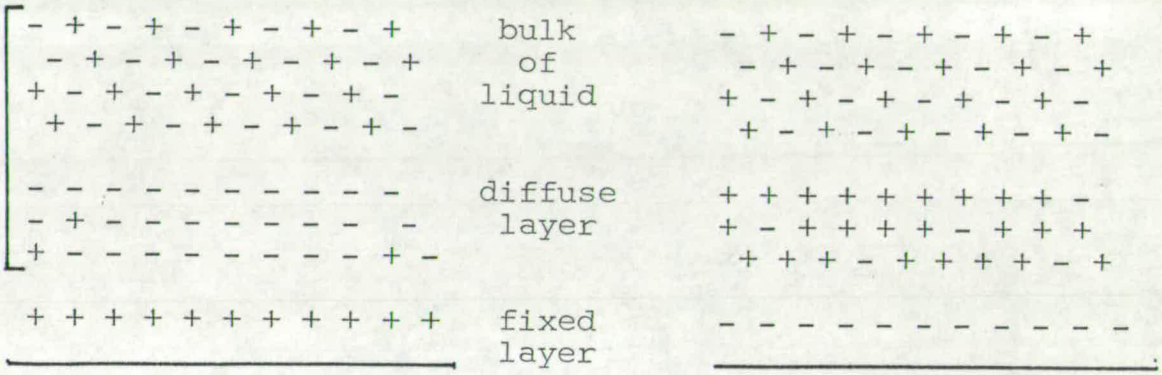



fig.1.1: Electrical double layer at liquid-solid interfaces in the sea.

Bracket shows the position between which the electrokinetic potential is thought to exist.

\+ : positively charged ions
\- : negatively charged ions

Left diagram: fixed layer formed by positive ions, hence relatively more negative ions in the diffuse layer.

Right diagram: fixed layer formed by negative ions, hence relatively more positive ions in the diffuse layer.

In the bulk of the liquid there are equal mumbers of positive and negative ions in both diagrams.

From Meadows & Anderson (1979).

& Marshall 1982a). Molecules in the interior of the solid will interact with molecules surrounding them, whereas those at the surface can only interact with those below them. Thus the surface free energy will tend to be greater than that of the interior of the solid. A tendency for reduction of the free energy at the solid-liquid interface favours bacterial attachment and this could be achieved by adsorption of a bacterial surface polymer on the solid surface by one of the previously mentioned interactions. Surface free energy is, in addition, a measure of the degree to which water can be adsorbed onto a surface, and is referred to by some authors as the substrate wettability (Dexter et al., 1975; Pringle & Fletcher, 1983).

The critical surface tension of a solid is an indirect measure of surface free energy, or wettability, and can be evaluated by making contact angle measurements. Dexter et al (1975), studying the influence of substrate wettability on the attachment of marine bacteria to various surfaces, including a range of plastics, metals and glass, clearly showed an influence of the critical surface tension of the surface on attachment. Maximal fouling was found to occur on high energy surfaces and minimal fouling on low energy surfaces. This work also suggested that the events occurring at the surface within the first few hours are critical, and are the most likely to be influenced by surface properties. Fletcher & Loeb (1979) measured contact angles and attached bacterial numbers on a range of solids and also found a direct correlation. Baier (1980) suggested that basic events of marine fouling, including initial microfouling and ultimate macrofouling, exhibit a general

dependency on critical surface tension. The contact angle method of determining surface characteristics has been used in a number of other studies (Fletcher, 1983; Fletcher & Marshall, 1982b; Pringle & Fletcher, 1983). Bright & Fletcher (1983) report that bacterial activity appears to promote firm attachment to surfaces, and that the attached bacterial activity depends on the properties of the surface, particularly wettability.

Recent evidence indicates that the degree of hydrophobicity of a surface may influence the attachment of marine bacteria to it. The majority of studies report a preference for hydrophobic materials (Fletcher, 1983; Fletcher & Loeb, 1979; Pringle & Fletcher, 1983). Since high energy surfaces are hydrophilic and low energy surfaces are hydrophobic, this is contrary to the work of Dexter et al. (1975). However, Absolom et al. (1983) suggest that the extent of attachment to a solid surface depends on the critical surface tensions of the bacteria and the suspending liquid involved, in addition to that of the surface itself. Thus, attachment is more extensive to hydrophilic substrata than to hydrophobic substrata when the surface tension of the bacteria is greater than that of the suspending medium. When the surface tension of the suspending liquid is greater than that of the bacteria, the opposite pattern occurs. These observations underline the possible involvement of a number of attachment mechanisms as suggested by Fletcher & Marshall (1982b); Loeb et al. (1983); and Paul & Jeffrey (1985), and emphasize that in considering the influence of surface free energy on bacterial attachment to surfaces, the system must be analyzed as a whole.

### 1.3.2 Physical Factors

In natural environments, in addition to the effects of the physicochemical characteristics of a surface, physical factors such as surface texture and hydrodynamic characteristics may be important in microbial attachment.

Electrostatic interactions with bacteria may be affected by undulations and projections on surfaces. Fletcher & Marshall (1982a) suggest that, as repulsion forces decrease with a decrease in the radius of curvature of an approaching surface, it may be easier for a bacterium to come into contact with a surface projection than with a relatively flat plane. However, using scanning electron microscopy and epiflourescence in studies on bacterial colonization of marine sand sediments, Weise & Rheinheimer (1978) found that protected sites i.e. crevices rather than ridges were favoured in the colonization process.

Fletcher & Marshall (1982a) also discuss the importance of hydrodynamic factors on microbial attachment. In most natural aquatic environments, water flow is turbulent, and an increase in turbulence results in an increased rate of microbial fouling. Bacteria are indirectly affected by an increase in turbulence due to the resultant increase in transfer of bacteria, nutrients and heat between areas of water. Thus surface texture can affect the degree of turbulence near the surface, but the major advantages of roughness are thought to result from an increased area for attachment, and by providing shelter from shear forces or abrasion (Weise & Rheinheimer, 1978).

Similarly, in studies on bacterial colonization of surfaces coated with antifouling paints, Dempsey (1981a,b) found that the porous nature of the matrix, formed by the dissolution of cuprous oxide particles within the insoluble paint matrix, allowed invasion by bacteria and the possibility of pore blockage by adhesive polysaccharides.

On the contrary, Berk et al. (1981) investigated microbial film formation on a variety of rough and smooth metal surfaces, including copper; aluminium; stainless steel; and titanium, and found that the morphology of the film formed depended, not on the texture of the metallic surface, but on its chemical composition. Thus, again it is difficult to generalize on the influence of surface characteristics on microbial attachment in natural waters, as matters are complicated by the many factors involved.

### 1.3.3 Surface-conditioning films

Deviations from the expected effects of surface free energy and charge on microbial attachment to solid surfaces may result from the masking of these characteristics by formation of a surface conditioning film. This film is formed by spontaneous adsorption of macromolecules, including proteins; glycoproteins; proteoglycans; and polysaccharides, which are present in low amounts in natural waters, and results in a new outer surface that is characterized by the properties of the external molecules of the adsorbed species. Thus, the presence of variable and undefined dissolved components in natural waters may alter surface characteristics and the rate of

attachment through adsorption to the surface (Fletcher & Loeb, 1979). Furthermore, the surface film may introduce lectin-like, bacteria-specific determinants that may bind selected bacterial species (Orstavik, 1977b).

Studies on the formation of conditioning films have shown the process to be influenced by the relative surface free energies (Baier, 1972; Dexter et al., 1975) and surface charges (Marszalek et al., 1979; Zaidi et al., 1984) of the surfaces involved. Since the first monolayer of adsorbed conditioning layer formed masks the original surface and its critical surface tension, the influence of the original surface characteristics on attachment must be indirect. Baier (1972) suggests that macromolecules are first adsorbed and then configuration conversions at the interface provide for strong binding with exuded polysaccharide components from the first arriving cellular species, allowing primary film formation. Fletcher & Marshall (1982b) indicated that the configuration of the adsorbed macromolecules differs on surfaces with different surface free energies, and that different side chains may anchor the macromolecules to different surfaces.

A number of studies have shown varying results on the influence of adsorbed organic films on microbial attachment. In some cases, surfaces with adsorbed protein inhibited attachment of bacteria. (Fletcher, 1976) showed that the attachment of a marine pseudomonad to polystyrene was inhibited by albumin, gelatin, fibrinogen and pepsin. Orstavik (1977b) demonstrated that albumin and concanavalin A impaired attachment of streptococci to glass. However, Meadows (1971) found that the influence of proteins on attachment to glass

surfaces depended on the charge of the protein at pH 7. Positively charged proteins, such as salmine, were found to reduce attachment, possibly by adsorbing to the bacterial and glass surfaces and reducing their negative charge and thus their tendency to interact. Negatively charged proteins enhanced attachment, but the mechanisms of this process are unclear. Fletcher & Marshall (1982b) showed that adsorption of proteins tended to reduce attachment of bacteria, though this differed considerably depending on the test surface and the nature of the protein. The reduction in attachment was attributed to changes in the surface free energy of the substratum due to the addition of the protein. Fletcher & Loeb (1979) suggest that adsorption of a protein at a surface makes it less favourable for attachment through steric effects or by affecting hydration of the surface and converting a favourable, hydrophobic surface to an unfavourable, hydrophilic one.

Conversely, Corpe (1970b) found that greater numbers of bacterial cells attached to polysaccharide-coated glass slides than to untreated surfaces. These differing results may indicate that the nature of the adsorbed organic matter affects the tendency to favour attachment, or that the quantity of the adsorbed film has some influence, as Baier (1972) reports that at no time do conditioning films cover the whole surface area. Wardell et al. (1983) suggested that small quantities of adsorbed material may promote the accumulation of bacteria at surfaces due to the increased nutritional status of the interface, whereas completely coated surfaces will have a modified surface charge which, depending

on the nature of the charge could aid or inhibit the attachment process.

In addition to the dissolved organic species, other dissolved substances, such as inorganic ions, have been found to affect microbial attachment through interaction with surfaces. The nature and concentration of such ions seems to have an important influence.

Monovalent (Orstavik, 1977a), divalent (Marshall et al., 1971a) and trivalent (Olson et al., 1976) cations have in some cases been found to promote attachment, whereas in the case of a marine pseudomonad, trivalent cations were found to inhibit attachment (Fletcher, 1980). Duddridge et al. (1981) report that a range of metal cations of differing valencies reduced microbial attachment at a concentration of 0.05M.

Divalent cations may favour attachment by implementing a decrease in the thickness of the electrical double layer (Marshall et al., 1971a), whereas trivalent cations may prevent attachment through interaction with and denaturation of any acidic surface polymers involved (Fletcher, 1980).

Variations in the effects of similar ionic species may be attributed to differences in the concentrations tested. Marshall et al. (1971a); Orstavik (1977a); and Gordon & Millero (1984) report that as electrolyte concentration increased up to 0.1M attachment increased, whereas at greater concentrations, attachment decreased. However, Stanley (1983) found that although results agreed up to concentrations of 0.01M, above this attachment decreased.

The role of conditioning films in microbial attachment to surfaces is not yet clear. Progress in this area is hampered by the divergent experimental designs investigating different species of bacteria, surface types and conditioning macromolecules. In addition, evaluation of adsorbed films presents some difficulties. Some techniques involve studies on the physicochemical properties of dried conditioning films (Baier, 1980). However, dried films may bear no relation to the surface in the aqueous environment as denaturation and configurational changes of the adsorbed macromolecules will result.

The bubble contact method used by Fletcher & Marshall (1982b) has the advantage that the aqueous phase is present, and, in the presence of macromolecules, the behaviour of the bubble may reflect that of a bacterial cell approaching a surface. This technique may be important in providing information on the role of conditioning films in attachment processes.

## 1.4 The Bacterial Cell Surface

A major requirement for microbial attachment to solid surfaces is some form of contact between the bacterial surface and the solid. Thus the strength of attachment may depend partly upon the structure and composition of the bacterial surface. This has been demonstrated by several workers. Abbott *et al.* (1983) found that two strains of *Streptococcus mutans*, differing in surface

properties, differed in their tendency to attach to solid surfaces. Fletcher (1983) demonstrated that the preincubation of bacteria with alcohols affected their subsequent attachment to surfaces and suggested that this may occur by affecting cell surface properties in some way. Dawson et al. (1981) suggest that the attachment process may be enhanced by a change in bacterial outer surfaces induced by a starvation stimulus. Bright & Fletcher (1983) found that the number of cell washings performed in experimental procedures had a considerable influence on attachment ability and suggest that this may be due to removal of cell surface components. In discussing the influence of bacterial surface characteristics on attachment, factors similar to those involved in the characterisation of solid surfaces must be considered. The physicochemical properties of the bacterial cell surface will be determined by the nature of the surface components.

### 1.4.1 Physicochemical Properties

Electrophoretic studies reveal that, at pH values normally encountered in natural environments, all bacteria possess a net negative surface charge to which surface components contribute (Marshall, 1980). The electrical double layer exists for bacterial surfaces as it does for solid surfaces. Kjelleberg & Hermansson (1984) studied the effects of changes in charge using electrostatic interaction chromatography, but were unable to make any generalizations on the relationship between bacterial surface charge

and binding to glass surfaces. However, variations in environmental factors such as nutrient availabilty and pH will influence the composition of bacterial surfaces and hence their net charge. In addition, the net negative charge of a bacterial surface may be modified by the adsorption of multivalent cations, organic materials or colloids present in natural waters (Marshall, 1980).

The surface free energy of a bacterial cell is also an important factor in attachment. To reliably predict surface-surface interations, the surface free energies of both the interacting surfaces are required. However, measurements of the surface free energies of bacterial cells are difficult to make and may be unreliable.

Dahlback et al. (1981) used hydrophobic interaction chromatography to evaluate the hydrophobicity of bacterial surfaces. These results indicate that this may be of importance in attachment as a higher proportion of hydrophobic bacteria were found at interfaces than in surrounding waters. Hydrophobic interaction chromatography was also the technique employed by Kjelleberg & Hermansson (1984), who showed a positive correlation between bacterial surface hydrophobicity and binding to glass surfaces in four marine isolates.

Another approach in evaluating bacterial surface hydrophobicity is to measure bacterial adherence to hydrocarbons, such as hexadecane. Correlations have been found between the adherence to hydrocarbons and attachment to other surfaces (Rosenberg, 1984).

### 1.4.2 Bacterial Surface Layers

Bacteria can be grouped into two types on the basis of their ability to stain in the Gram-reaction. This reaction reflects differences in the chemistry and arrangement of the wall structures between the two groups. Those of Gram-positive bacteria are simple in structure, while those of Gram-negative bacteria are more complex, having a multi-layered structure . This is reflected in the chemical composition of the walls (table 1.1) and in the location of the polymers (fig.1.2). The cytoplasm of both types is enclosed by the cytoplasmic membrane.

### 1.4.3 The Cytoplasmic Membrane

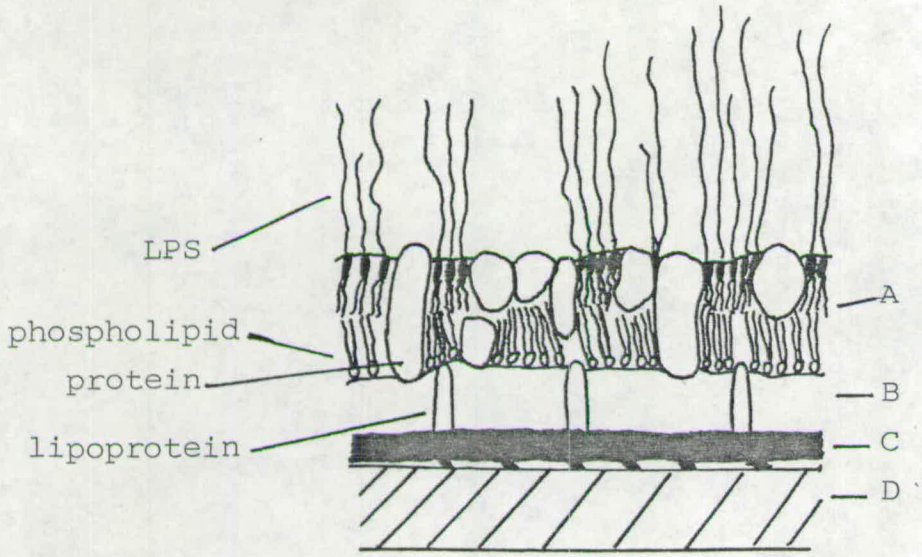
The cytoplasmic membrane consists of a bilayer of phospholipids with polar groups facing outwards into the aqueous phase at the membrane surface, while the hydrophobic fatty acid chains extend into the centre of the membrane. The membrane proteins, which may account for more than half of the membrane dry weight are intercalated into the phospholipid bilayer. A schematic account of the organization of the unit membrane is given by the fluid mosaic model of Singer & Nicolson (1972).

The cytoplasmic membrane has a variety of functions. These include the maintenance of osmotic gradients, solute transport and the organization of cell wall synthesis. In addition, the cytoplasmic membrane may be involved in the organization of

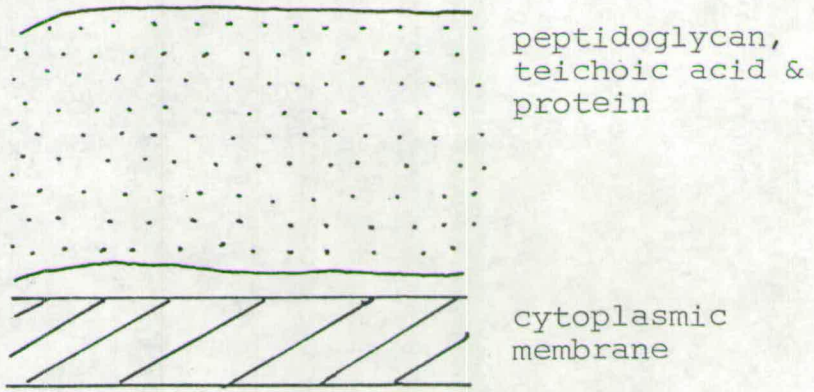
| ORGANISM TYPE | PEPTIDOGLYCAN | TEICHOIC ACID | LPS | LIPID | PROTEIN |
|---|---|---|---|---|---|
| Gram +ve | 40-50% | + | - | 2% | c.10% |
| Gram -ve | 5-15% | - | + | 20% | c.60% |

Table 1.1: Composition of bacterial cell walls (from Dawes & Sutherland (1976)).

fig.1.2: Location of Cell Wall Polymers in Bacteria.




a. Diagrammatic representation of the Gram-negative cell envelope.
From Ward & Berkeley (1980).
A: outer membrane
B: periplasmic space
C: peptidoglycan
D: cytoplasmic membrane




b. Diagrammatic representation of the Gram-positive cell wall.
From Dawes & Sutherland (1976).

chromosome segregation and cell division and is the site of oxidative metabolism and energy generation.

### 1.4.4 The Peptidoglycan Layer

Peptidoglycan is found in all bacteria, with the exception of Mycoplasmas and Archaebacteria, and is the main structural component of the cell wall. In Gram-positive bacterial walls, this polymer may account for as much as 80% of the total weight, though values of 40-50% are more commonly found. In Gram-negative organisms it only accounts for around 5-15% of the total weight of the envelope.

Peptidoglycan consists of glycan chains, containing alternating residues of N-acetyl glucosamine and N-acetyl muramic acid in a β,1-4 linkage. Separate chains are cross-linked to varying degrees by short peptide bridges attached to muramic acid residues.

In Gram-negative bacteria, the peptidoglycan is present as a single layer within the outer membrane (fig.1.2), which will prevent it from reacting with macromolecules in the external environment. This is in contrast to Gram-positive bacteria, which have some peptidoglycan exposed at the surface.

### 1.4.5 The Gram-positive Cell Wall

In addition to peptidoglycan, Gram-positive cell walls contain a variety of other polymers, including teichoic or teichuronic acids, polysaccharides and proteins. Teichoic acids are probably

the second most abundant secondary wall polymer isolated from Gram-positive bacteria, and are highly negatively charged polymers. When phosphate is a limiting component in the growth medium, teichoic acids are replaced by teichuronic acids, which are also negatively charged. Both teichoic and teichuronic acids are covalently linked by phosphodiester bonds to muramyl residues in peptidoglycan (Ward & Berkeley, 1980). Ellwood et al., (1982) speculate that this switch from teichoic to teichuronic acids could result in alterations in the charge distribution on the cell surface, which may affect attachment properties. In addition, the net charge of teichoic acids is modified by the presence of D-alanine groups, the amount of which is known to vary with growth conditions.

Lipoteichoic acids are a second form of teichoic acid found in Gram-positive bacteria. These are not covalently linked to peptidoglycan, but are intimately associated with the wall, and are anchored in the cytoplasmic membrane. Lipoteichoic acids have been implicated in the adhesion of streptococci to epithelial cells (Beachey & Simpson, 1980), and appear to penetrate the thickness of the wall and become exposed at the cell surface.

Polysaccharides, which are covalently linked to peptidoglycan; and proteins, in close association with the wall, but not linked, may form antigenic determinants on the cell surfaces of some Gram-positive bacteria.

In bacteria not producing capsules these various polymers may represent the outer surface of the cell and thus be in direct

contact with the environment.

### 1.4.6 The Gram-negative Cell Envelope

Gram-negative bacteria are more commonly found in natural waters than Gram-positives, and their envelope is more complex than the Gram-positive bacterial cell wall. The peptidoglycan is located in the region between the cytoplasmic membrane and the outer membrane, described as the periplasmic space. The periplasmic space constitutes around 20-40% of the total cell volume and is distinguished by a distinct ionic composition and the possession of a unique series of proteins. The outer membrane, like the cytoplasmic membrane, has the appearance of a unit membrane, and consists of three major components: lipopolysaccharide (LPS); protein; and phospholipid. LPS is contained exclusively in the outer leaflet of the membrane, and can be divided into three distinct regions. A complex lipid (Lipid A) is linked via a $C_8$ sugar acid, KDO to a core polysaccharide, which is in turn substituted by side chains of oligosaccharides (O-side chains). The O-side chains extend from the bacterial surface and exhibit gross differences in composition and structure from one group of organisms to another, and often within a single species. Clearly, the length of the O-side chains projecting from the surface will be of importance in any possible interactions with another surface (Ward & Berkeley, 1980). Ellwood *et al.* (1982) speculate that the balance of positively and negatively charged substituents in the LPS

moiety, which will be influenced by changes in environmental conditions, may determine net charge and thus affect attachment properties. Pringle et al. (1983) found that mutants of a strain of Pseudomonas fluorescens showing increased attachment levels had 40-50% less polysaccharide in their LPS as compared to wild-type cells. They suggest that increased attachment may be due to a reduced coverage of the cell surface by the O-side chains, leaving many of the surface proteins and lipids exposed.

A number of proteins are present in the outer membrane. Major outer membrane proteins include the porins, which form hydrophilic channels and have a non-specific role in the passage of small molecules across the membrane. Also present is another major protein, the lipoprotein. This either exists freely or is covalently linked to the peptidoglycan, thus anchoring the outer membrane to the peptidoglycan layer. Minor proteins are found with a low number of copies per cell, and may serve as high affinity transport systems for nutrients or as receptors for bacteriophages or colicins. Proteins such as these must be available to the external environment (Ward & Berkeley, 1980).

The role of outer membrane proteins in attachment is unclear. Pringle et al. (1983) found small changes in the outer membrane proteins of mutants exhibiting greater attachment than wild-type organisms, but observed no changes in the major outer membrane bands. Ward & Berkeley (1980) speculate that specific mannose lectins isolated from surface layers of E. coli may be present as components of the outer membrane.

## 1.4.7 Extracellular Polysaccharides

Many bacteria produce an extracellular polysaccharide, either as a discrete capsule or as a layer of loosely attached slime. This can be greatly influenced by changes in environmental conditions. The nature of the linkage of this exopolysaccharide to the underlying wall structure is unknown, but might be due to the presence of lipid intermediates, involved in synthesis, at the growing ends of polymer chains. Ward & Berkeley (1980) suggest that these lipids might anchor the polymers in the bacterial membrane.

Bacterial exopolysaccharides can be divided into homopolysaccharides, having only one type of sugar residue, or heteropolysaccharides containing more than one type of residue. Homopolysaccharides include glucans, which are polymers of glucose; and levans, which are polymers of fructose. However, the majority of bacterial exopolymers are heteropolymers often containing acetyl groups, pyruvyl groups or uronic acids. The latter two substituents confer an overall negative charge on the polymer (Ward & Berkeley, 1980). The polysaccharides of individual bacteria may have unique physical properties, and if the capsular polysaccharide has a different chemical character from that of the surface of the organism, the overall surface charge of the cell will be modified (Corpe, 1970a).

By staining submerged slides, Zobell (1943) demonstrated the

involvement of a secreted cementing substance in microbial attachment. Several authors have reported definite involvement of polysaccharides in the attachment of bacteria to surfaces (Allison & Sutherland, 1984; Corpe, 1970b; Fletcher & Floodgate, 1973; Johnson et al., 1977). However, Corpe et al. (1973), using polysaccharide-less mutants of a marine bacterium, found that the polysaccharide was not required for attachment, though, because of its surface charge and other properties, it served as a polymeric bridge for other cells. Pringle et al. (1983) suggest that exopolysaccharides may not be concerned with the primary attachment of the producing cells but with the development of the subsequent bacterial film.

Ward & Berkeley (1980) speculate that the capsular material of bacterial cells may be specifically involved in attachment by means of direct interactions with lectins on the surface. This is analogous to the trifoliin A-binding capsule of Rhizobium trifolii (Dazzo & Brill, 1979; Dazzo et al., 1982), which carries determinants showing highly specific antigenic cross-reactions with a surface component on clover seedling roots.

Little work has been carried out on the chemical and structural properties of polysaccharides involved in attachment. Sutherland (1980) demonstrated that there was no single chemotype of polysaccharide associated with attached aquatic bacteria and suggested that adhesiveness is associated with polysaccharide structure rather than composition. Improved analytical procedures should aid in structural studies on microbial adhesive

polysaccharides.

In addition, the ecological role of adhesive polysaccharides requires further study (Sutherland,1982) to determine the possible function of such polymers in nutrient adsorption and the trapping of ions.

### 1.4.8 Surface Appendages

Scanning electron microscopy has shown that, in addition to capsules and slime layers, many bacteria have varied physical means of attachment, including flagella; pili or fimbriae; and adhesive stalks and holdfasts (Dempsey, 1981a; Paerl, 1975). These surface appendages serve as anchors, and the energy expenditure required for their formation is rewarded by the increase in growth and replication on favourable physical and chemical substrates.

Bacterial flagella, the organelles involved in chemotaxis and movement, have been implicated in attachment. Meadows (1971) demonstrated that when bacteria attach to glass slides they often do so as if attached at one end of their cell by their flagella. However it is possible that the cells could be attached by other surface components, such as adhesive polysaccharide, located at the poles. Fletcher (1979b) found that removal of flagella by treatment with a blender reduced attachment. Again this evidence is inconclusive as blending may also have removed other surface components. Corpe (1980) speculates that the role of flagella in attachment may be to permit chemotactic responses to nutrients at

surfaces and to help the cell stay in the proximity of the surface until firm attachment occurs.

Fimbriae (or pili) are filamentous appendages frequently found on the surfaces of Gram-negative bacteria, and have been clearly implicated in attachment in some cases. For example, Duguid (1959) showed that fimbriate, but not non-fimbriate strains, of Klebsiella had adhesive properties. The net surface charge of fimbriae is lower than that of the outermost cell wall layer, and this reduces the problems bacteria have in overcoming electrostatic repulsion by enabling the cell to make contact with the surface while held at a distance by the repulsion barrier (Wardell et al., 1983). In addition, fimbriae may increase the active surfaces of the cell. Ottow (1975) suggests that this may enhance respiration and nutrient uptake, but this correlation is difficult to explain.

Later in the development of bacterial films, bacteria with stalks and holdfasts appear. Henrici & Johnson (1935) described a number of stalked bacteria attached to submerged slides and proposed a new order, Caulobacteriales. Poindexter (1964) described the biological properties of this group and showed by electron microscopy that the wall of the stalk is continuous with the cell wall and is not a secreted material. At the end of the stalk is a holdfast material which is secreted and implements attachment. Umbreit & Pate (1978) have characterized the holdfast region of Asticacaulis biprosthecum and suggest that an extracellular polymer at the holdfast region is responsible for attachment and is at least in part an acidic polysaccharide.

Filamentous sheathed bacteria of the genus Sphaerotilus and other Chlamydobacteriales show ability to attach to solid surfaces (Mulder & van Veen, 1963). The presence of specific holdfasts; the sticky character of the sheath; and perhaps the ability of the organisms to accomodate imperfections of a rough surface may all contribute to their tendency to attach.

Gliding bacteria of the genus Saprospira in natural environments may become attached to glass slides and show long filaments with some type of holdfast structure (Lewin, 1965).

Corpe (1970a) discusses the possibility of the involvement of inorganic "cements" in attachment. An example of this is described by Mulder & van Veen (1963) during investigations on the Sphaerotilus-Leptothrix group. When Sphaerotilus natans is in the presence of ferrous iron, ferric hydroxide may be deposited in and on its sheaths, and there may also be participation in the oxidation of manganous compounds to give $MnO_2$ precipitates. The binding or encircling of cells or their appendages with a solid surface gives the view that these deposits are acting as mineral cements.

## 1.5 Attachment Mechanisms

Studies by Marshall et al. (1971a) on the mechanism of the initial events in the attachment of marine bacteria to surfaces confirmed the suggestion of Zobell (1943) that the attachment process consists of two initial phases. Bacteria were found to be first attracted to a surface and, after several hours, became firmly

attached. The first stage is a reversible stage and involves the deposition of cells on the surface. This is often referred to as the adsorption step. A prerequisite for this initial phase is the transport of cells to the surface, which controls the initial rate of deposition. Reversible attachment may then be followed by the irreversible phase, which is time dependent and involves permanent attachment to the surface. A final phase involves the development of a thick film of bacteria leading perhaps to colonization by larger organisms.

### 1.5.1 Transport of Cells to Surfaces

The transport of microbial cells to solid surfaces is described by Characklis & Cooksey (1983) and involves diffusion, gravity, taxis and fluid dynamic forces. Eighmy et al. (1983) investigated wastewater biofilm formation and found that bacterial attachment required cell transport to the surface by either mobility or fluid eddies. Floodgate (1972) suggests that bacterial cells may respond to chemoreceptors on surfaces. In studies on bacterial chemotaxis, Young & Mitchell (1972) found that attachment to surfaces can be significantly enhanced by the presence of attractants.

The role of chemotaxis in the first stages of attachment indicates that the presence of flagella will have a strong influence. Evidence to confirm this comes from the work of Fletcher (1979b) and Stanley (1983) who showed that loss of motility led to a decrease in the rate of attachment. Motility may assist by one of two processes (Fletcher, 1980): by increasing the force

with which a bacterium encounters the surface, or by increasing the statistical chance of the bacterium encountering the surface.

More recently, however, it has been indicated that microorganisms are transported to surfaces solely by hydrodynamic forces (Lewin, 1984). It appears that, relative to the distance that bacteria swim in their random walk mode of mobility, the nutrient gradient clings very close to the surface and thus cannot influence the direction of an organism's movement.

### 1.5.2 Reversible Attachment

#### 1.5.1.2 DLVO Theory

Marshall et al. (1971a) found that the numbers of attached bacteria increased with increasing electrolyte concentration, as the thickness of the electrical double layer decreased. In addition, reversible attachment occurred at lower concentrations of divalent electrolytes than of monovalent electrolytes, which was thought to be related to the greater compression of the double layer in the divalent system. It was suggested that this phenomenon could be explained by the Derjaguin & Landau and Verwey & Overbeek (DLVO) theory of colloid stability. This states that the total interaction energy ($V_t$) of two smooth particles is determined by the sum of van der Waals attractive energy ($V_a$) and the usually repulsive electrostatic energy ($V_r$) resulting from the overlapping ionic atmospheres (diffuse double layers) around the surfaces. The magnitude of repulsion will depend on the size of the individual net

surface charges and will increase with a decrease in distance between the two surfaces. Repulsion also depends on the radii of curvature of the surfaces and the electrolyte concentration (Fletcher & Marshall, 1982a). With a decrease in radius of curvature there is a decrease in repulsion. With an increase in electrolyte concentration, repulsion forces are reduced due to the adsorption of ions counterbalancing the net charge of the surface (fig.1.3). As mentioned previously, the thickness of the electrical double layer is reduced with increasing electrolyte concentrations. For example, it is 200Å at 0.2mM NaCl, but at 0.2M NaCl it is reduced to about 7Å (Meadows & Anderson, 1979). Seawater has an electrolyte concentration approximately equivalent to 0.6M NaCl, which suggests that electrostatic repulsion may be more significant in freshwaters than in seawater.

Curves showing the variation of $V_t$ with separation distance (h) show two values of h at which net attraction occurs (fig.1.4). These are referred to as the primary minimum (h very small) and the secondary minimum (h=5-10nm). These are divided by an area of separation where repulsion forces are high and difficult to overcome.

Marshall et al. (1971a) suggest that during the reversible phase in seawater it is likely that bacteria are attracted to the point of the secondary minimum. However, the kinetic energy of the motile marine pseudomonad used in these studies could not overcome the repulsion barrier, and the magnitude of the attractive energy at the secondary minimum was not sufficient to hold the bacteria against the shearing effect of seawater. This may be the basis for

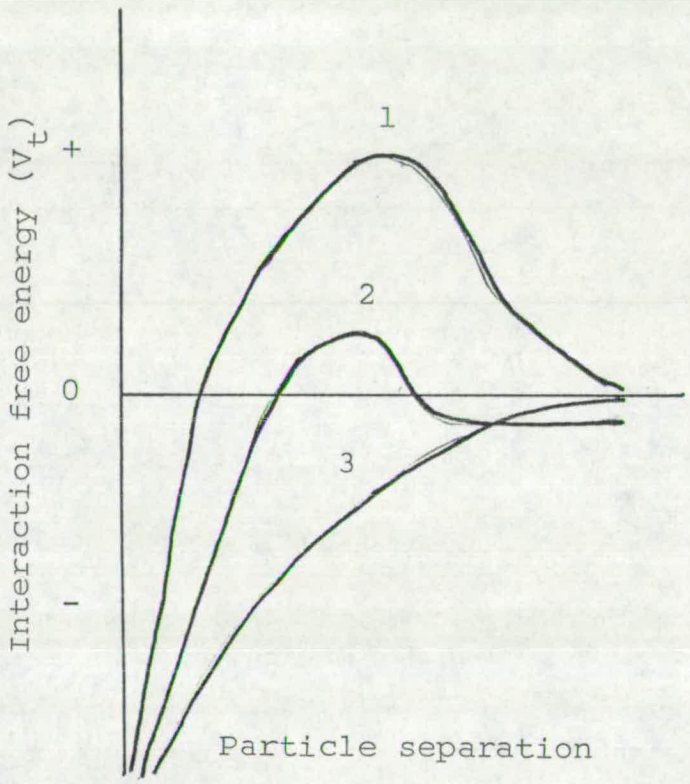



fig.1.3: Interaction free energy ($V_t$) vs separation as a function of electrolyte concentration at low (1), intermediate (2) and high electrolyte concentration From Fletcher & Marshall (1982a).

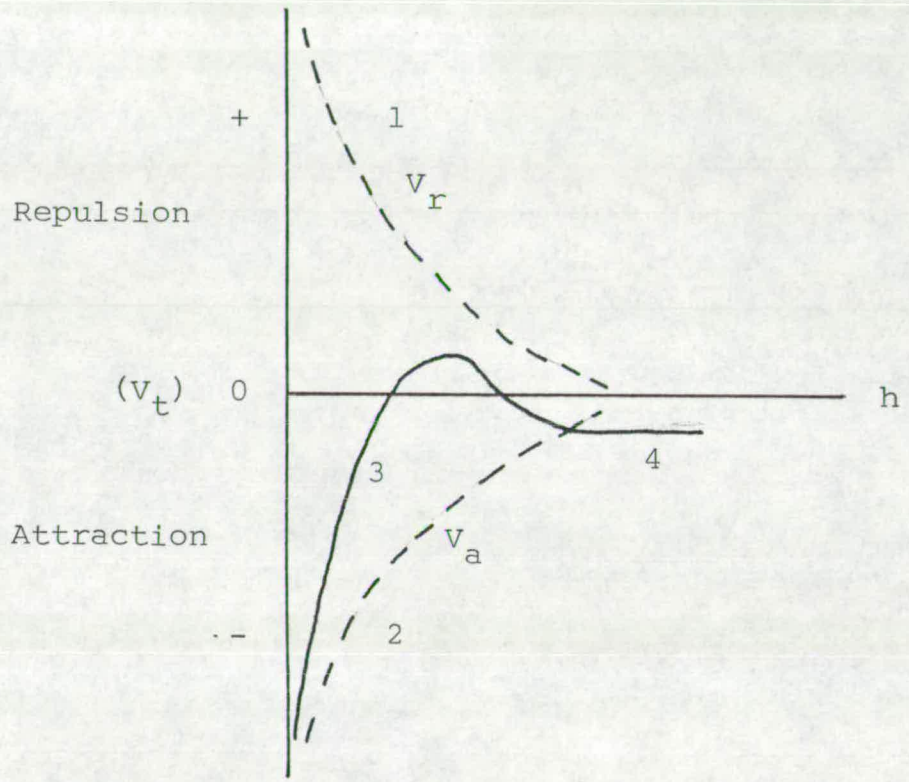



fig.1.4: Variation of the total interaction energy ($V_t$) with h, the separation distance between a particle and a surface.
1: repulsion curve
2: attraction curve
3: primary minimum
4: secondary minimum
$V_t$ is obtained by summation of $V_r$ and $V_a$.

the reversible attachment of cells, where the cells exhibit Brownian motion and are easily removed by washing.

The predictions of the DLVO theory on the repulsion between bacterial and solid surfaces must be treated with caution. Firstly, the DLVO theory assumes that interacting surfaces are smooth planes. However , this is not the case, and contact may thus be at a single point or at many points. At these points, short-range forces (see Section 1.5.2.2) may be stronger than long range forces. The complex nature of the cell surface, comprising several layers and often covered by long-chain capsular polymer allows for various types of interaction to take place simultaneously (Tadros, 1980). Secondly the studies by Marshall et al. (1971a) only took account of the adsorption mechanisms of two marine species, making generalizations on the initial events of attachment difficult. Finally, the results mentioned were obtained in systems with little or no fluid shear stress, a situation which rarely occurs in the natural environment.

### 1.5.2.2 Short-range Forces

At short separation distances between surfaces and bacterial cells, short-range forces are of importance. These include a number of interaction types: chemical bonds, such as electrostatic, covalent and hydrogen bonds; dipole interactions; and hydrophobic bonding (Tadros, 1980). These short-range forces may be repulsive or attractive depending on the nature of the surfaces involved.

When both surfaces are hydrophilic, a short-range repulsion force results; when both are hydrophobic the short-range interaction is a net attraction (Rutter & Vincent, 1980).

Hydrophobic bonding has recently been implicated as an important attachment mechanism (Dahlback et al., 1981; Fletcher & Loeb, 1979; Paul & Jeffrey, 1985). Hydrophobic interactions occur in aqueous solutions between non-ionic, non-polar surfaces, as, in spite of weak van der Waals interactions, strong interactions between water molecules exclude the hydrophobic surfaces from the aqueous phase.

### 1.5.3 Irreversible Attachment

As cells approach a surface, van der Waals attractive forces can hold them in the weak DLVO secondary minimum for a short time during which irreversible attachment can occur. Irreversible attachment depends on the physicochemical adsorption of bacterial cell surface components onto the surface, accompanied by a reduction in free energy within the system (Fletcher & Marshall, 1982a). Irreversibly attached cells do not show Brownian motion and are not easily removed by washing (Marshall et al., 1971a). This phase is characterized by the appearance of extracellular polymeric adhesives which are either able to adsorb non-specifically onto the surface or enter into specific interactions with complementary polymers adsorbed onto, or forming part of the surface. This process is termed polymeric bridging and may be able to overcome the repulsion barrier.

The presence of these bacterial surface polymers has been demonstrated by electron microsopy (Corpe et al., 1973; Eighmy et al., 1983; Fletcher & Floodgate, 1973; Marshall et al., 1971a), which suggests that cell surface polysaccharides are the bacterial adhesives. However, Corpe et al. (1973) showed that the acidic polysaccharide of a marine pseudomonad was not required for attachment to occur, suggesting that other mechanisms may be involved. Ellwood et al. (1982) found that some surface-associated microbial communities did not depend on the production of large amounts of extracellular polysaccharide for attachment. Fletcher & Marshall (1982b) demonstrated that some proteases are able to remove attached bacteria, suggesting that proteins may be involved. This was also demonstrated by a reduction in attachment due to the presence of inhibitors of protein synthesis (Fletcher, 1980; Paul & Jeffrey, 1985).

In some cases, attachment mechanisms may depend upon the presence of cations which could bridge or screen negative charges on the bacterial or solid surface, and thus reduce repulsion forces. Marshall et al. (1971a) found that omission of divalent cations ($Ca^{2+}$ & $Mg^{2+}$) prevented irreversible sorption to surfaces. Few bacteria attached in media lacking these ions, and those which did showed no evidence of polymer production. However, Fletcher (1979) showed that an increase in trivalent cations ($La^{3+}$ &$Al^{3+}$) had a detrimental effect on attachment of a marine pseudomonad, probably due to some interaction between the ions and the adhesive polymer.

The situation is complicated by the effects of changes in environmental conditions, which may alter the quantity or

composition of bacterial surface polymers (Fletcher & Marshall, 1982a). It is uncertain whether these polymers are preformed or formed as a result of de novo synthesis. The work of Marshall et al. (1971a) suggests that the polysaccharide material involved in the attachment of a marine pseudomonad was preformed, as bacteria leaving the surface at the reversible stage left behind some material at the attachment point. This is confirmed by the work of Stanley (1983), who found that cells in suspension were prepared to adhere, although further synthesis of extracellular adhesive material probably occurred after attachment.

Floodgate (1972) and Fletcher & Floodgate (1973) describe two types of polysaccharide produced by marine bacteria. The primary polysaccharide appeared to be preformed during growth and not formed as a response to settlement. However, as cells begin to replicate and form microcolonies, the extracellular polysaccharide changed into a more reticulate form. This secondary polysaccharide probably developed from the primary polysaccharide and eventually replaced it. These studies suggest that attachment of bacteria does not initiate de novo synthesis of adhesive polymers, but promotes the development of a reticular secondary polysaccharide from a preformed primary polymer.

The best evidence in support of a requirement for de novo synthesis for attachment comes from studies on Streptococcus mutans. Mukasa & Slade (1973) report that the dextran-levan polymer involved in attachment of S. mutans to glass surfaces must be synthesized on the cell surface in order to participate in the attachment mechanisms. Hamada (1977) showed that preformed,

cell-associated glucan of S. mutans had no significant adherent properties. Attachment occurred as a result of active de novo glucan synthesis in the presence of sucrose, cells and extracellular and/or cell-bound glucosyl transferases.

This work also provided evidence that some sort of physiological activity may be required for the attachment process. Fletcher (1980) suggest that this might involve motility and the synthesis and secretion of polymers or enzymes involved in polymer bridging. The involvement of physiological activity was investigated in this study using inhibitors of energy production and protein synthesis. Results indicated that attachment could be energy dependent and that protein synthesis could be involved. However the requirement was shown to vary with the nature of the surface. Results have shown that surface-associated bacterial populations have a higher metabolic rate than suspended bacteria (Ellwood et al., 1982; Hendricks, 1974; Kinner et al., 1983). Bright & Fletcher (1983) suggest that bacterial activity is important both in initial attachment and ability to remain attached to a surface. Strengthening of attachment may also be related to cell activity.

Apart from extracellular polysaccharides, several other mechanisms are known to be involved in the irreversible stage of attachment. These are specific mechanisms found in bacteria attaching to specific surfaces, and involving interactions between complementary molecular structures on the bacterial surface and the attachment surface. Such mechanisms involve specialized appendages such as fimbriae, stalks and holdfasts, and sheaths. Also involved may be lectins or antigenic interactions.

1.5.4 Microbial Film Development

The final stage in the attachment process is the growth and development of attached cells, leading to the production of microcolonies and eventually film formation. In natural environments, this film would be composed of a variety of microorganisms. During film development, results have shown that substantial amounts of polymer are formed (Dempsey, 1981a,b; Ellwood et al., 1982; Fletcher & Floodgate, 1973).

The sequence of attachment of different bacterial types was observed on glass slides and electron microscope grids in seawater by Marshall et al. (1971b). After a one hour exposure period, mainly rod-shaped bacteria had attached. Coccoidal and spiral forms attached after about six to eight hours, stalked bacteria at around twenty four hours, and after five day's exposure, diatoms and protozoa were found to have attached. It was not indicated from this study whether selection in the attachment process is in the initial attraction or firm adhesion phase. However, small rods appeared to have some selective advantage in irreversible attachment. As repulsion forces decrease with a decrease in the radius of curvature of the approaching particles (Fletcher & Marshall, 1982a), repulsion will be reduced with smaller cells, which thus may be able to overcome the repulsion barrier more easily than larger cells.

The composition of the microbial film becomes increasingly complex with time and varies with the nature of the initial surface (Dempsey, 1981a,b; Marszalek et al., 1979). Environmental conditions influence the thickness of the biofilm, as reported by

Bott et al. (1983) relative to the effects of climatic conditions on biofouling in an industrial cooling water system.

### 1.5.5 Environmental Factors Affecting Attachment

Clearly, environmental factors greatly influence the attachment of microbial cells to solid surfaces. Duddridge et al. (1981) studied the effects of changes in the environmental conditions on the adhesive properties of a common aquatic bacterium, Pseudomonas fluorescens. Attachment of the organism to stainless steel was found to be highly dependent on the suspended cell concentration, the time allowed for attachment, temperature of attachment, culture age, culture growth substrate, pH and ionic composition of the suspending medium, and surface shear stress. Similar environmental factors have been discussed in reports by Abbottet al. (1983); Characklis (1973); and Stanley (1983).

The number of environmental factors which exert an influence on microbial attachment further complicates the difficulties already mentioned (Section 1.3) in making simple generalizations on the attachment of bacteria to solid surfaces in natural environments.

## 1.6 Techniques Used in Studies on Microbial Attachment to Surfaces

The ability to determine the importance of attached microbial populations in numerical and functional terms, and to investigate the mechanisms involved in microbial attachment has depended on the development of techniques for demonstration, enumeration and

recovery of the bacterial cells comprising these populations.

### 1.6.1 Materials Used in Examination of Attached Microbial Populations

The use of submerged glass slides is the traditional method of examining attached populations of fresh and marine waters (Henrici & Johnson, 1935; Zobell, 1943; Zobell & Allen, 1935), and continues to be used for isolation and examination of natural microbial populations (Corpe, 1970b; Lewin, 1965) as well as for experimental studies on the physiological basis of microbial attachment (Marshall et al., 1971a; Meadows, 1971).

Other materials used in examination of microbial attachment include: a range of plastics (Dexter et al., 1975; Fletcher, 1976, 1977, 1983; Fletcher & Loeb, 1979); metals (Berk et al., 1981; Dexter et al., 1975; Fletcher & Loeb, 1979; Marszalek et al., 1979); surfaces coated with marine antifouling paints (Dempsey, 1981a,b); and bacteriological filter membranes (Fletcher & Floodgate, 1973).

### 1.6.2 Microscopy

For demonstration of the existence of attached microbial films on immersed surfaces, microscopy has proved a sound and widely used method. However, each type of microscopy has its advantages and disadvantages. Thus, for the best results a combination of techniques should be used.

1.6.2.1 Light Microscopy

For immersed glass slides, ordinary light microscopy combined with an appropriate staining method is useful. Allison & Sutherland (1984) have developed a method which allows differential staining of the bacterial cells and the extracellular polysaccharide, implicating a role for exopolysaccharide in attachment and build up of microbial films.

Phase contrast microscopy observes living bacteria and avoids loss of cells during fixing and processing. It also provides a topographic "face" view of large areas of colonized surfaces (Costerton, 1980). Meadows (1971) used phase contrast microscopy and dark ground optics to observe the attachment of bacteria to glass slides.

In studies which have relied on light microscopy for the examination of microorganisms attached to opaque or organic materials, the use of special fluorescent staining and optical techniques was required (Maigetter & Pfister, 1975; Obuekwe et al., 1981; Weise & Rheinheimer, 1978; Wiebe & Pomeroy,1972). Another technique for the microscopic examination of the fouling communities of submerged opaque surfaces was developed by Sechler & Gunderson (1971, 1972). Attached microbial communities were removed intact from a variety of test surfaces in a Parlodian (nitrocellulose) film, then stained and mounted for microscopy, allowing qualitative and quantitative differences to be followed.

1.6.2.2 Electron Microscopy

A disadvantage of light microscopy is that it is limited to low resolution, which is insufficient to reveal fine structural details of attached organisms which may be involved in the attachment process. This shortcoming has promoted widespread use of electron microscopy, together with special preparative techniques which minimize disturbances of microbial arrangements. Costerton (1980) discusses the advantages and disadvantages of scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM has proved an excellent technique for examining the microbial colonization of a wide range of surfaces immersed in the sea (Berk et al., 1981; Dempsey, 1981a,b; Dexter et al., 1975; Paerl, 1975), and in other systems (Eighmy et al., 1983; Kinner et al., 1983; Ridgeway & Olson, 1981). This technique shows great promise in providing a means for the study of the character of solid surfaces and for relating the distribution of bacteria to the topography of the surface (Weise & Rheinheimer, 1978). However, the limitations of SEM mean that is unable to detect bacterial colonization of interior surfaces and, due to limited resolution, may fail to show up the fine extracellular structures that mediate attachment.

The superior resolution of TEM, coupled with the use of specific stains, reveals these important structures in fine detail. Direct immersion of microscope grids has been used to study attached bacteria with TEM (Marshall et al., 1971b). This technique has been useful in the detection of the relationship of attached bacteria with the surface and with each other. Specific

staining methods have been used to yield a chemical characterization of certain structures. Alcian blue and ruthenium red have been used to demonstrate the involvement of an extracellular, compact acidic polysaccharide in bacterial attachment to surfaces (Corpe et al., 1973; Fletcher & Floodgate, 1973). However, it should be recognized that staining of this type is not necessarily an indication that the polysaccharide surrounding the cells is acidic, as ruthenium red is also known to stain some neutral polymers (Abbott et al., 1980).

### 1.6.4 Enumeration of Attached Bacteria

Enumeration of attached bacteria has been achieved by a range of techniques. Simple methods involve plate counts (Dempsey, 1981b) or direct microscopic counts (Fletcher, 1977). However, plate counts may be unreliable due to clumping of cells, and direct microscopic counting proves very laborious. These methods involve the physical removal of attached microcolonies from the surface, either by scraping or by other physical means, which is also used in the isolation of attached species (Corpe, 1970b; Dempsey, 1981b). During this procedure bacterial cells may be lost due to breakage or incomplete removal. Thus enumeration by these techniques represents only a minimum of the attached population (Costerton, 1980) and gives no information on the microenvironmental relationships between different components of the biofilm, or on the relationship between distribution and the physical features of the surface (Meadows & Anderson, 1979). These problems also apply to the method of Fletcher (1983) and Pedersen (1982a,b), where the numbers of bacteria were correlated to

an absorbance measurement of the surface film.

Orstavik (1977a,b) overcame these difficulties by using radioactive labelling techniques to enumerate the attachment of Streptococcus faecium to glass surfaces. Another way to eliminate the problems is to use indirect chemical methods in the quantitation of biomass on surfaces. Dexter et al. (1975) measured biomass by a lipopolysaccharide test utilising Limulus lysate. Berk et al. (1981) used levels of extractable lipid phosphate and total extractable palmitic acid to quantitate biomass, and found that community composition of a biofilm can also be assayed by biochemical methods such as evaluation of the relative proportions of various types of fatty acids. White (1983) used assays of cellular components found in specific subsets of the microbial community to determine the composition of biofilms. Dahlback & Pedersen (1982) and Pedersen (1982a,b) followed total biofilm development by evaluating the increase in dry weight of the biofilm and by measurement of protein content, and determined the viability of the biofilm by measurement of adenosine nucleotides.

The composition of biofilms can also be measured by immunofluorescence techniques (Zambon et al., 1984). This involves the isolation of individual attached species, the production of specific antibodies to them, and the coupling of these to fluorescent compounds which are then used to identify cells of the species in mixed natural populations by microscopy.

Mackowiak & Marling-Cason (1984) performed a comparative analysis of in vitro assays of bacterial adherence, including microscopic, quantitative subculture and radiolabel assays. It was

recommended that to obtain a comprehensive picture of bacterial attachment, more than one assay system should be used.

### 1.6.5 Continuous Culture

A useful technique to aid in the understanding of microbial attachment to surfaces and, in particular, to study the influence of different variables on this, is continuous culture (Baltzis & Fredrickson, 1983; Ellwood et al., 1982; Hendricks, 1974; Larsen & Dimmick, 1964; Maigetter & Pfister, 1975). This technique is discussed by Wardell et al. (1983), who suggest that it has a distinct advantage as the attachment and growth of cultures may be studied over an extended time period, which is not possible in batch culture experiments.

Simple mathematical models can be used in such studies, and involve three basic assumptions: that bacteria attach to the walls in contact with the culture as a monolayer; that, after an initial build up period, the attached bacterial mass remains constant and progeny are released into the culture; and that the growth rate of the attached bacteria is the same as that of the suspended population. However, experimental results often differ from those predicted by theory as bacteria do not always grow as a monolayer, the biomass does not always remain constant, and the attached populations may grow at different rates due to increased nutrient concentrations at surfaces. Hendricks (1974) reports that deviations from theoretical predictions in chemostat studies are especially noticeable when nutrient concentrations are high, but

less apparent with low nutrient concentrations. This suggests that, by virtue of the low nutrient concentrations present in natural waters, application of continuous culture under the same low nutrient conditions may be a relevant method for studies on attachment in these areas.

In some studies, a range of experimental apparatus has been devised to determine the effects of various parameters on microbial attachment in specialized systems. Dahlback & Pedersen (1982) and Pedersen (1982a,b) studied the formation of a microbial film on glass surfaces arranged in lamellar piles parallel with circulating seawater. This system was developed with particular reference to the flow conditions used in electrochemical concentration cells. Bott et al. (1983) inserted test plates in a cooling water circuit under controlled flow rate conditions to study biofouling of an industrial cooling system. A rotating disc technique has also recently been used in studies on microbial attachment (Abbott et al., 1983; Kinner et al., 1983).

### 1.6.6 Genetic Studies

In order to understand the processes that regulate bacterial adhesion, genetic studies have recently been undertaken (Simon et al., 1985). The regulation of the attachment processes will be genetically controlled. The gene products of the chemotaxis and motility systems may allow bacteria to find a surface. Genes that code for a variety of organelles and surface components are required for adhesion. Other genes are involved in the production of

extracellular polysaccharides which form the matrix in which bacteria are maintained on a variety of surfaces. By defining these genes and processes, it is hoped that a molecular understanding of attachment processes will emerge and will aid in the development of procedures to control microbial biofouling.

## 1.7 Control of Marine Fouling

To combat the problems caused by fouling of surfaces immersed in seawater, it is desirable that some means of controlling microbial film formation be devised. In closed systems, such as seawater conduits and cooling systems of industrial plants, power stations and ships, chlorine and its derivatives have been used successfully to control microbial fouling, as discussed by Meadows & Anderson (1979). Berk et al. (1981) and Caron & Sieburth (1981) studied the effectiveness of mechanical brushing in removing surface films. It was found that this method selectively removes components of the attached community and changes the community composition of the residual film, resulting in increased extracellular polymer production.

In areas of open waters, the most common method of protection of exposed surfaces is by coating with antifouling paints. Paints containing compounds of copper, mercury and arsenic have been widely used for antifouling protection (Meadows & Anderson, 1979), but efforts to produce more active and longer lasting antifouling paints have led to the development of organometallic antifoulants such as triphenyltinfluoride (Dempsey, 1981a,b). Surfaces coated with

these paints seem to delay the later stages of the microbial fouling succession.

Organotin compounds are known to be highly toxic to Gram-positive bacteria, but virtually ineffective against Gram-negative bacteria (Dempsey, 1981a,b). As most fouling bacteria are Gram-negative, paints containing organotin as the sole toxicant will have relatively little impact on the fouling community.

There are several other disadvantages connected with the use of antifouling paints. Starkey (1957) discusses the susceptibility of matrix constituents of antifouling paints to microbial attack in seawater. This affects the liberation of the toxic component which prevents fouling, and reduces the period of antifouling action. In addition, the matrices of the paints may include insoluble polymers which are used as a carbon source by bacteria, thus contributing to rapid release of the toxicant from the paint (Meadows & Anderson, 1979). The porous nature of the paint matrix allows invasion by bacteria and the possibility of pore blockage by extracellular polysaccharide, leading to a loss of antifouling efficiency (Dempsey, 1981a,b). In addition, attached bacteria may be responsible for the breakdown of antifouling compounds to non-toxic metabolites.

The effects of antifouling paints seems to be mainly on the later stages of fouling, as microorganisms are more resistant to antifouling agents than their macroscopic counterparts. In particular, some primary film forming organisms have been found to develop a tolerance to copper compounds (Dempsey, 1981a,b), which

underlines the fact that bacteria have a capacity to develop mutant strains capable of growth in the presence of previously toxic compounds. Selective pressure ensures the survival and multiplication of such naturally occuring mutants. Dempsey suggests that, due to the development of tolerance to copper and other heavy metals by bacteria, and to the ineffectiveness of organotin compounds against Gram-negative bacteria, compounds able to prevent growth, or polymer production, should be developed and incorporated in antifouling paints to extend their service life.

Dexter et al. (1975) suggest the possibility of controlling primary film formation by manipulation of initial surface properties rather than by using coatings with leachable toxicants. Many other workers have shown that the tendency for bacteria to attach is dependent on surface composition (Baier, 1972, 1980; Berk et al., 1981; Fletcher, 1979a, 1980; Fletcher & Loeb, 1979; Marszalek et al., 1979; Pringle & Fletcher, 1983). The importance of the critical surface tension, wettability or hydrophobicity has been discussed by Dexter et al. (1975) and Fletcher & Loeb (1979), and the influence of chemical composition has been discussed by Berk et al. (1981). Gordon et al. (1981) studied the effect of polarization on the attachment of marine bacteria to copper and platinum surfaces. Attachment to copper was enhanced by cathodic polarization and reduced by anodic polarization. The latter effect may be due to an increased generation rate of divalent copper ions at the surface. Both anodic and cathodic polarization of platinum inhibited attachment. Thus this may be useful as an antifouling method. More detailed information on the effects of surface

properties on conditional film formation, on the rate of surface colonization, on the successional sequence of microorganisms and on the actual strength of microbial attachment would be useful in assessing methods of controlling microbial fouling. Consideration of the problem from this approach has led to the development of a new coating system to control marine fouling (Shell UK Ltd., 1985: Aquatect 1). This is based on a silicon rubber polymer combined with a special exuding oil. This combination creates a surface whose free energy is in the critical range which makes attachment by marine organisms difficult. This method has clear advantages over traditional antifouling coatings. Firstly, both of the prime constituents of the coating are non-toxic and non-irritant thus, unlike conventional antifoulants, they are environmentally acceptable. In addition, they are more easily applied and have a longer effective working life.

Chet et al. (1975) and Young & Mitchell (1972) studied the relationship between negative chemotaxis and the prevention of microbial film formation on surfaces exposed to seawater, with a view to using organic repellants in non-toxic concentrations to control marine fouling. The most effective repellants were found to be acrylamide and benzoic and tannic acids, which yielded significant control of slime formation, even after forty days of immersion in seawater. This negative chemotactic response of bacteria allows them to move away from an unfavourable environment, even when a high concentration of nutrients is present on the toxic surface. This then provides an alternative method of controlling marine fouling to the commonly used methods which involve toxic

compounds that may be hazardous to the biological equilibrium in the marine environment. Repulsion provides a promising new approach to fouling control using non-toxic chemicals.

The wide variety of bacteria present in natural waters causes difficulties in proposing solutions to the problems of marine fouling. The wide differences in the observations of various workers on the effects of surface characteristics on attachment emphasize this difficulty (Dexter et al., 1975; Fletcher & Loeb, 1979).

## 1.8 Effects of Heavy Metals and Organometallic Compounds on Microorganisms

The use of antifouling paints depending on heavy metals and organometallic compounds as toxicants has led to the development of fouling organisms which are tolerant to these agents (Dempsey, 1981a,b). Determination of the mechanisms of action of toxicants on marine bacteria, and of the mechanisms of tolerance to these toxicants may aid in the development of antifouling measures.

### 1.8.1 Binding to Bacterial Surface Components

A prerequisite for the uptake and subsequent action of toxicants on the bacterial cell will be some form of interaction with the cell surface components. Metal binding appears to be at least a two step process (Beveridge & Murray, 1980), in which the first step is a stoichiometric interaction between metal and

reactive chemical groups in the wall structure. The next step is an inorganic deposition of increased amounts of metal, which can be readily discerned by electron microscopy (Beveridge, 1977; Beveridge & Murray, 1976). A number of studies have attempted to locate the cell surface components involved in metal deposition.

The Gram-positive cell wall has been compared to a low-density, ion exchange resin which can freely exchange ions with the environment (Marquis et al., 1976). Heptinstall et al. (1970) investigated the ability of bacterial cell walls to bind magnesium ions. This study suggested that teichoic acids are responsible for cation binding in Gram-positive cell walls, and that a major function of these polymers is to maintain a high concentration of divalent cations in the region of the membrane. Divalent cations are required for the function of membrane enzymes and to maintain the physical integrity of the membranes.

However it has been suggested that the walls of Gram-positive bacteria offer a number of potential sites for metal binding (Beveridge, 1977; Beveridge & Murray, 1976, 1980). These include the phosphodiester groups of teichoic acids, the free carboxyl groups of peptidoglycan, and, to a lesser extent, the sugar hydroxyl groups of both wall polymers and the amide groups of peptide chains. Beveridge & Murray (1980) found that chemical modification of the carboxyl groups of the wall had the most profound effect on metal deposition. These are present on the glutamic acid residues, on unlinked diaminopimelic acid and terminal D-alanine residues of peptidoglycan. Beveridge & Murray (1980) suggest that it may be the constituent glutamic acid of Bacillus subtilis peptidoglycan

that is the most potent metal binding component in the wall. Doyle et al. (1980) studied the chemical basis for the selectivity of metal ions by the cell wall of B. subtilis, and found that different cations, and protons, compete for common binding sites.

The binding of metals to the cell envelopes of Gram-negative bacteria has been examined by a number of techniques. These include phcsphorous nuclear magnetic resonance (Ferris & Beveridge, 1984; Strain et al., 1983), electron microscopy (Hoyle & Beveridge, 1983) and atomic absorption and X-ray fluorescence spectroscopy (Beveridge & Koval, 1981). The latter techniques were used to test the binding of over thirty metals to cell envelopes of E. coli. Results suggest that most metal deposition occurred at the polar head group regions of the constituent membranes or along the peptidoglycan layer.

The peptidoglycan of all Gram-negative bacteria is of the same chemotype as that of B. subtilis and should thus be a potent site for metal deposition. Hoyle & Beveridge (1984) found that it proved able to bind substantial amounts of metallic ions from aqueous solution. The anionic carboxyl groups of the D-glutamic acid residues of the peptide stems have been implicated as sites of metal binding, although other sites may also be involved.

Recent studies have shown that isolated outer membranes from E. coli are capable of sequestering metal cations from aqueous solution (Ferris & Beveridge, 1984; Hoyle & Beveridge, 1983). Electron microscopy of metal-loaded samples revealed an aggregative depositon of metal on one surface of the membrane. This indicates that at least one distinctive binding site is asymmetrically arranged in

outer membrane vesicles. Heptinstall et al. (1970) have suggested that LPS, which possesses sugar residues, phosphate groups and basic residues, may serve to bind metal ions. NMR spectroscopy of LPS indicates that the glycosidic diphosphate moiety (Lipid A) participates in a high affinity binding site (Strain et al., 1983). Acidic groups on exposed polypeptides in the outer membrane may also be involved in interaction with metals (Beveridge & Koval, 1981).

Extracellular polymers produced by many microorganisms are capable of binding cations, thus making them more readily available to the cell surface for transport into the cell (Cassity & Kolodziej, 1984; Rorem, 1955). Corpe (1975) found that copper taken up by marine bacteria was concentrated in the cell surface layers. The extracellular polymer used in these studies formed insoluble precipitates with soluble salts of iron, copper and lead, and formed non-diffusible complexes with cobalt, nickel, zinc, calcium and magnesium. There was no evidence that the marine bacteria investigated produced larger quantities of polymer when grown in the presence of added metal than in its absence. However, Dempsey (1981a,b) reports that, in the presence of copper-based antifouling paints, bacteria produce copious amounts of slime material. It is not clear though whether the presence of copper induces tolerant bacteria to secrete excessive amounts of polymer or selects for excessive polysaccharide-producing strains. Exopolymers from a freshwater sediment bacterium were evaluated by a combination of equilibrium dialysis and atomic absorption spectrometry and were found to bind copper (Mittleman & Geesey,

1985).

Copper binding properties of natural ionic polysaccharides, including bacterial polysaccharides, have been examined by Manzini et al. (1984) and Reisenhofer et al. (1984). All polymers investigated gave rise, in aqueous solution, to complexes with divalent copper ions. The mode and extent of the interaction depended on several factors: the nature of component sugars and their relative distribution in the chain; the copper to polymer ratio; the polymer concentration; and the ionic strength.

### 1.8.2 Action on the Bacterial Cell

The effect of various metal cations on bacteria has been studied. Failla (1977) suggests that divalent copper ions present at a concentration of less than 100μm, and divalent zinc ions present at a concentration of less than 10μm may act on bacterial membranes by binding to respiratory chain components, thus inhibiting respiration by blockage of electron transfer. Schreiber et al. (1985) also indicate that addition of copper to cultures of a marine vibrio inhibits respiration. These authors propose that there is more than one toxic effect of copper, and that toxicity is affected by the presence of oxygen. In aerobic culture, a single cytotoxic effect is implicated. Increased toxicity under anaerobic conditions is attributed to a second cytostatic effect. The site of action in this case may be a pathway involved in the anaerobic metabolism of glucose. Under anaerobic conditions, the sulphydryl groups in proteins could be increased, and it is known that copper

ions bind readily to these groups. Such binding could disrupt the tertiary structures of proteins thus giving another possible mechanism for increased toxicity. Corpe (1975) also suggests that heavy metal cations may interact with proteins, such as transport proteins, rendering them non-functional.

The effect of nickel ions on a marine bacterium, Arthrobacter marinus, has been investigated by Cobet et al. (1970). They produced a state of unbalanced growth resulting in large, plasmolysed sphaeroplasts. It was speculated that this effect involved cell division mechanisms, inhibiting multiplication while the cells continued to grow. This would result in osmotic irregularity and a plasmolysed appearance. Schreiber et al. (1985) indicate that copper ions may also inhibit cell division. Arcuri & Ehrlich (1977) suggest that divalent copper and nickel ions may attack at sites related to cell growth, such as sites of active cell wall synthesis. Exposure of cells to copper may result in an increase in permeability (Schreiber et al., 1985). Beveridge & Murray (1976) indicate that metal ions may cause a change in the porosity of the plasma membrane thus affecting the permeability of this. The toxicity of copper could also be attributed partly to its ability to compete with the transport systems of other metals, essential for metabolic processes. Jasper & Silver (1977) found that a number of metal ions were all transported by the same system, and increased concentrations of one ion inhibited uptake of the others.

There is little information available on the mechanism of action of organotin antifouling compounds. However, Dempsey

(1981a,b) indicates that, although these compounds are highly toxic to Gram-positive organisms, they are ineffective against Gram-negative species. It is likely that organotin antifoulants do not reach toxic levels within Gram-negative cells, as more water-soluble organotin compounds are effective. This thus suggests that the outer membrane layer acts as a penetration barrier against such hydrophobic compounds.

### 1.8.3 Bacterial Tolerance

Bacterial tolerance to some heavy metals can be controlled by genes on extrachromosomal resistance factors or plasmids which can also control antibiotic resistance. The penicillinase plasmids of Staphylococcus aureus carry determinants of resistance to a series of inorganic ions as well as resistance to penicillin (Novick & Roth, 1968). Heavy metal resistance in Alcaligenes eutrophus was found to be plasmid-encoded, though no antibiotic resistance was found to be associated with these plasmids. Recent reports have shown an inducible plasmid-determined resistance to copper among isolates of E. coli (Rouch et al., 1985; Tetaz & Luke, 1983). The plasmid involved is designated pRJ1004 and is conjugative. Plasmids have also been implicated in determination of resistance of pseudomonads to organic tin compounds (Anisimova et al., 1985). However, determinants of resistance to some heavy metals may be chromosomally-encoded (Calomiris et al., 1984; Novick & Roth, 1968) or transposon-encoded (Robinson & Tuovinen, 1984; Rouch et al., 1985; Williams & Silver, 1984).

Plasmid-mediated metal resistance may be ecologically important since resistance can be rapidly transferred from resistant to sensitive bacteria. Thus populations in natural habitats may adapt genetically to conditions of metal toxicity faster than by processes of spontaneous mutation and natural selection.

Heavy metal tolerance in bacteria can occur by a variety of mechanisms, as discussed by Gadd & Griffiths (1978). Microbial hydrogen sulphide production often has significant effects on metal toxicity since most heavy metals form insoluble sulphides with hydrogen sulphide. Consequently, hydrogen sulphide-producing organisms often exhibit tolerance to heavy metals. Production of organic substances that bind or chelate heavy metals can markedly affect toxicity. Physical sequestration of heavy metal by the outer layers of bacterial cells will effectively remove it from the environment and thus reduce its toxicity. Many of the components of the bacterial surface layers are known to bind metals (see Section 1.8.1). Bacterial polysaccharides may be able to chelate metal ions (Forster & Lewin, 1972; Rorem, 1955), and Dempsey (1981a,b) suggests that the production of copious amounts of extracellular polysaccharide may afford some protection to bacteria colonizing copper-based antifouling paints.

Another mechanism of tolerance is the transformation of metals, resulting in their detoxification. This method has been studied chiefly with mercury, and involves reduction, oxidation and methylation reactions, with the mercury ultimately being volatilized from the medium (Gadd & Griffiths, 1978). In addition, some resistant bacteria detoxify organomercurial compounds by a lyase

reaction (Williams & Silver, 1984).

Decreased uptake or impermeability to a metal may be a means of resistance. Lutkenhaus (1977) showed that selection for resistance to divalent copper ions in E. coli led to the loss of a major outer membrane protein. It was suggested that copper penetrates the outer membrane through hydrophilic pores formed by this protein to reach a sensitive site within the cell. In the absence of this protein, it is possible that copper may enter through other pores at a lower rate, resulting in reduced accumulation. Rouch et al. (1985) confirm the possibility of reduced accumulation, and propose that the plasmid-encoded copper resistance mechanism in E. coli involves an intracellular binding protein coupled with an efflux mechanism which ejects excess copper. This system could function as a copper homeostasis mechanism which enables the resistant cells to tolerate concentrations of copper which are toxic to normal cells, while still allowing operation of copper-dependent functions within the cell.

### 1.8.4 Influence of Environmental Factors on Heavy Metal Toxicity

Most studies on the effects of toxicants on microorganisms have been carried out under artificial laboratory conditions. However, in the natural environment the response of a microbe to a toxicant is dependent on numerous factors. Thus, in determination of heavy metal toxicity, the potential effects of the physicochemical characteristics of the environment must be considered.

Firstly, the pH of the environment can have a considerable

effect on the chemical form and solubility of heavy metals. This will determine the availability and thus the toxicity of the metals.

In general, at an acid pH metals exist as free ionic cations, but at alkaline pH, such as in seawater, the ionic cations precipitate as insoluble hydroxides or oxides (Gadd & Griffiths, 1978). Thus low pH generally increases the availability of metal cations whereas high pH decreases availability and thus toxicity. However, some studies have shown that, as pH increased the toxicity of divalent metal cations also increased, as discussed by Babich & Stotzky (1980). There are two possible explanations for this phenomenon. The first is that as pH is increased, the concentration of divalent metal ions decreases as the hydroxylated monovalent species is formed. Monovalent cations presumably penetrate biological membranes more readily than do divalent cations, thus the enhanced toxicity may have reflected a greater uptake. Secondly, increased toxicity at high pH may have reflected competitive antagonism between hydrogen ions and cations on the cell surface. As pH was increased and the concentration of hydrogen ions decreased, competition for sites on the cell surface would be reduced.

Whereas pH is an indication of the availability of hydrogen ions in a system, the Eh, or oxidation-reduction potential is a measure of the availability of electrons. Positive Eh values indicate an oxidative environment and negative Eh values indicate a reducing environment. The valency of many heavy metals is dependent on the Eh of the environment and, as differently charged forms of the same element may exert different effects on microorganisms, Eh will be of importance (Babich & Stotzky, 1980).

As Eh and pH are related, the ionic form of heavy metals is influenced jointly by the availability of electrons and hydrogen ions in the environment. Copper, for example, disappears from solution at any Eh when the pH is greater than 6, and at an Eh below +200mV when the pH is less than 6 (Gadd & Griffiths, 1978). Thus these two factors should be considered jointly.

The toxicity of heavy metals can be affected by the presence of other ions in the environment. In general, cations tend to reduce heavy metal inhibition (Babich & Stotzky, 1980; Gadd & Griffiths, 1978), presumably by an antagonistic mechanism. The protective effect of one cation on the toxicity of a second heavy metal cation is probably a reflection of competition for sites on the cell surface, with a reduction in the accumulation of the heavy metal within the cell.

Anions are able to reduce heavy metal toxicity by formation of complexes with, and thus removal of the toxicant from the environment. In natural environments, different negatively charged ligands, each present at a different concentration and having a different affinity for the same cation, compete for the heavy metals (Babich & Stotzky,1980). In seawater, both hydroxide and chloride ions compete for the same metals, and different complexes exert different influences on the microbial populations. In addition, other anions such as sulphide, carbonate and phosphate, interact with heavy metals to form insoluble compounds. This will decrease the availability and hence the toxicity of the heavy metal to the microbial populations. For example, the addition of phosphate decreased the toxicity of divalent copper ions to Aerobacter

aerogenes, presumably due to the formation of an insoluble complex (MacLeod et al., 1967).

One of the most important factors in determining the biological availability of a metal in a system is its binding to other environmental constituents which may result in a decrease in toxicity. Clay minerals, such as montmorillonite and kaolinite, have surfaces which are predominantly negatively charged and to which charge compensating cations are adsorbed. These cations are not permanent components of the clays and are constantly being exchanged for other cations in the environment. The total amount of exchangeable ions that a clay can retain is termed the cation exchange capacity. Heavy metals released into the environment can thus be exchanged for cations on the exchange complex of such clays, thereby removing the toxicants at least temporarily from solution and reducing their uptake by and toxicity to microorganisms (Babich & Stotzky, 1980).

The cation exchange capacity is also important in the effect of hydrous metal oxides, such as hydrous ferric oxide, manganese dioxide and aluminium oxide, on heavy metal toxicity. These hydrous metal oxides exhibit selective affinities for heavy metals and function in the same manner as clay minerals, removing the toxicant from the environment.

Organic matter, a relatively stable by-product of the microbial transformation of a variety of dead cells and extracellular products of various organisms, is also of importance in reducing the toxicity of heavy metals by binding to them (Babich & Stotzky, 1980; Gadd & Griffiths, 1978). This may occur by coordination bonding (as in

chelation); by covalent bonding to some functional groups, such as sulphydryl groups; or by cation exchange. Slowey & Jeffrey (1967) indicate that up to 50% of the total copper present in seawater exists in a complexed form, and that this complex is of a lipoidal nature.

Compounds which can chelate metals may also have a significant effect on toxicity to microorganisms when included in growth media. Toxic effects of copper were prevented, or reduced, in Aerobacter aerogenes by addition of yeast extract and cysteine (MacLeod et al., 1967), and in E. coli by addition of casein hydrolysate, yeast extract or peptone (Jones, 1964). This could be attributed to the ability of these compounds to bind copper, which has been demonstrated by Ramamoorthy & Kushner (1975).

There is little information available on the influence of temperature on heavy metal toxicity. However, strains of Staphylococcus aureus, with and without plasmids conferring resistance to cadmium, did not take up cadmium at 4°C. At 37°C, only plasmid negative strains took up substantial amounts (Kondo et al., 1974). This may be a reflection of greater resistance to cadmium at lower temperatures, possibly due to a change in membrane fluidity, or merely of a reduction in metabolic activity at the lower temperature.

Recent studies have indicated that hydrostatic pressure influences the sensitivity of marine bacteria to heavy metals. Arcuri & Ehrlich (1977) found that the effect on heavy metal toxicity varied with the type and concentration of toxicant tested and with the organism used. It was observed that, in some

organisms, increasing hydrostatic pressure over a range of 1-340 atm. may totally reverse trends in metal ion effect from toxic to protective to toxic. This indicates that abrupt changes in cell response may be taking place at critical hydrostatic pressures rather than gradual changes with increasing hydrostatic pressure. These authors suggest that the abrupt changes in response to metal ions at different pressures could be the result of pressure-induced, conformational changes in cell wall or membrane proteins, or intracellular structures such as ribosomes, assuming these to be the sites of action of metal ions. Such changes could cause different reactive groups to be made available for interactions with the metal ions.

The numerous environmental factors involved underlines the difficulties in assessing the toxicity of heavy metals to microorganisms. Clearly great care must be exerted to minimize interference from undefined constituents in media used in studies on microbial resistance to heavy metals. In addition, in considering the influence of the various environmental factors on heavy metal toxicity, the relative importance of each must be evaluated as interactions may occur.

# CHAPTER TWO: MATERIALS & METHODS

## 2.1 Isolation and Maintenance of Bacteria

Bacteria were isolated from seawater yeast extract agar plates (Table 2.1) which had been inoculated with seawater samples; with swabbings from natural marine surfaces, such as rocks and seaweed; or with swabbings from glass slides (76x26mm) or marine antifouling paint coated slides that had been immersed in running seawater for 7 days. All samples were collected from Port Seton, near Edinburgh, at low tide. Isolates were maintained on seawater nutrient agar (SWNA) slopes at $4^{o}C$ and also by lyophilization.

## 2.2 Marine Antifouling Paints and Toxicants

Marine antifouling paint samples S1, S2 and S3 (Table 2.2) were supplied by Hempel Technology, Copenhagen, Denmark.

Copper sulphate ($CuSO_4.\ 5H_2O$) and both inorganic and organic tin chlorides were tested as toxicants.

## 2.3 Growth Conditions

Bacteria used in experiments were routinely grown in a liquid culture medium prepared in artificial seawater (ASW, Table 2.3). This medium was designated Medium II Yeast Extract (MIIYE, Table 2.5). Cultures were grown in 100ml volumes in 250ml flasks and in 1L volumes in 2L flasks. Flasks were incubated on a rotary shaker (120 $rev.min^{-1}$) at $30^{o}C$ or at an appropriate temperature.

Table 2.1: Seawater Yeast Extract (SWYE) Agar

| | |
|---|---|
| Yeast Extract | 1g |
| Casamino acids | 1g |
| Oxoid agar No.3 | 20g |
| Filtered seawater | 1l |
| Glucose | 20g* |

* autoclaved separately

Table 2.2: Marine Antifouling Paints

| Sample | Toxicants used (approx.%) | | |
|---|---|---|---|
| | $Cu_2O$ | ZnO | Organotin |
| S1 | 25 | 11 | 0 |
| S2 | 0 | 21 | 24 |
| S3 | 30 | 3 | 11 |

Table 2.3:Artificial Seawater

| | |
|---|---|
| NaCl | 23.476g |
| $Na_2SO_4$ | 3.917g |
| $NaHCO_3$ | 0.192g |
| KCl | 0.664g |
| KBr | 0.096g |
| $MgCl_2 . 6H_2O$ | 10.626g |
| $CaCl_2 . 2H_2O$ | 1.459g |
| $SrCl_2 . 6H_2O$ | 0.040g |
| $H_3BO_3$ | 0.026g |
| Distilled water | 1l |

Table 2.4: Salts A

| | |
|---|---|
| $K_2HPO_4$ | 100g |
| $KH_2PO_4$ | 100g |
| Distilled water | 1l |

Table 2.5: MediumII-Yeast Extract (MIIYE)

| | |
|---|---|
| Yeast extract | 1g |
| Casamino acids | 1g |
| ASW | 500g |
| Distilled water | 500ml |
| Glucose | 20g* |
| Salts A | 20ml* |
| pH | 7.2 |

* autoclaved separately

Toxicants to be added to the medium were sterilized separately by autoclaving, in the case of copper sulphate or by membrane filtration (pore size 0.45µm; Millipore, France), in the case of organotin compounds. Paint-coated surfaces were sterilized by immersion in 0.5% (v/v) glutaraldehyde for 10 min. and subsequently rinsed in sterile distilled water (x5).

## 2.4 Characterization of Bacteria

### 2.4.1 Morphological and Physiological

Isolates were characterized by a range of features including: Gram-staining reaction; motility; presence of extracellular capsules or slime; production of glycogen; haemagglutination; and growth over a range of temperatures. These tests were performed mainly as described in Cruickshank (1965).

The presence of glycogen inclusion granules was determined by preparation of wet films in Gram's iodine and viewing by phase contrast microscopy.

Haemagglutination tests were carried out as follows. Horse red blood cells were separated from fresh blood by centrifugation and washed twice in physiological saline (0.9% w/v). A red blood cell suspension (3% v/v) was prepared in physiological saline and dispensed in equal volumes in the wells of a haemagglutination tray. The isolates tested were grown overnight in static culture, washed and resuspended in physiological saline. Equal volumes of each isolate were dispensed into separate wells containing red blood

cell suspension. The tray was agitated to mix the suspensions and incubated at room temperature for 2h, then overnight at 4°C. Results were read after each incubation period.

### 2.4.2 Biochemical

Isolates were characterized by a range of biochemical tests, including analysis of end products, enzymic activities and carbohydrate fermentations. These tests were carried out mainly as described in Cruickshank (1965). Production of amylase and lipase was determined on SWYE plates containing 1% starch and Tween 80 respectively.

## 2.5 Selection of Copper Resistant Mutants

Individual colonies of isolate 82Q grown on SWYE agar were picked off using sterile toothpicks and streaked onto a master plate of SWYE agar. This was incubated at 30°C until growth was observed on all streaks. Replica plating was performed onto SWYE agar containing a range of concentrations of copper sulphate. Replica plates were incubated at 30°C for 4d after which copper resistant mutants were selected and subsequently maintained in similar conditions to the original isolates.

## 2.6 Growth Curve Analysis

1ml aliquots of overnight cultures were added to 100ml of fresh medium (1% v/v inoculum) and incubated under routine growth conditions. 1ml samples were removed aseptically at 30 min. intervals and cell density was monitored by absorbance at 600nm. In addition, viable cells were enumerated as described below.

## 2.7 Preparation of Non-growing Cell Suspensions

100ml aliquots of overnight cultures were added to 1L of fresh medium (10% v/v inoculum) and grown until the appropriate phase of the growth cycle was reached. Cells were harvested by centrifugation (24000g; 30 min.: Sorval RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments, Delaware, USA), and resuspended in an appropriate volume of ASW, to which had been added 2% (w/v) glucose and 2% (v/v) Salts A. All steps were performed under aseptic conditions.

## 2.8 Attachment Assays

Chemically clean, sterile glass slides (76x26mm) were suspended in 200ml of non-growing, logarithmic phase cell suspensions in 250ml flasks and incubated on a orbital shaker (120 rev.min$^{-1}$) at 30$^{o}$C for the appropriate period of time. Slides were subsequently removed,

rinsed wih 50ml sterile ASW and swabbed with a sterile cotton wool swab moistened in ASW. Swabs were agitated in ASW and attached cells were enumerated as described below. Enumeration of cells in suspension was performed before and after incubation. The reverse sides of the glass slides were stained as described below.

## 2.9 Enumeration of Bacterial Cells

Viable cells in growth curve analyses and attachment assays were enumerated by serial dilution in sterile ASW and subsequent plating onto SWYE agar. Plates were incubated for 3 days at 30°C, after which counts were performed.

## 2.10 Visualization of Attached Bacteria

### 2.10.1 Light Microscopy

Cells attached to glass slides were stained by a method described by Allison & Sutherland (1984), which allowed visualization of associated exopolymers. Slides were covered with 10mM cetylpyridinium chloride (cetavlon) and air-dried for 30 min. at 37°C. After fixing by gentle heating and allowing to cool, slides were stained for 20 min. with a 2:1 mixture of 10% (v/v) Tween 80 and saturated aqueous congo red solution. The slides were then rinsed gently with tap water, stained for 2 min. with 10% (v/v) carbol fuchsin, rinsed and air-dried at 37°C. Viewing was by x1000 oil immersion. Photography was carried out on a Lietz Orthoplan

microscope with a camera attachment (Wetzlar, FRG) using Kodak Ektrachrome 160 film.

2.10.2 Scanning Electron Microscopy

Chemically clean, sterile glass coverslips (13mm diameter) were suspended in 100ml of a bacterial culture and incubated under routine conditions overnight. Coverslips were subsequently removed and rinsed in sterile ASW.

Fixation and visualization were carried out as a standard protocol (Glauert, 1975; Robards, 1978). Samples were fixed for 2h in 0.1M cacodylate buffer (pH 7.2) containing 2.5% (v/v) glutaraldehyde and 3.0% (w/v) tannic acid, then washed for two 10 min. periods in distilled water. This was followed by 2h fixation in 2% (v/v) osmium tetroxide in 0.1M cacodylate buffer and a further two 10 min. distilled water washes. Dehydration occurred through an acetone series (10%; 20%; 40%; 60%; 80%; 90%; 100%; 100%; 100%) for a 5 min. period in each. Samples were then subjected to critical point drying (Polaron critical point drier, Watford, England) using acetone, and $CO_2$ as the transitional fluid, attached to stubs using double sided tape and sputter coated with gold for 2 min. at 20mA deposition (Emscope SC500 modular sputter coater, Kent, England).

Attached cells were observed using an accelerating voltage of 40kV in a Cambridge Stereoscan 250 scanning electron microscope (Cambridge, England). Nikon 35mm film was used for photography.

## 2.11 Exopolysaccharide Preparation

### 2.11.1 Production

750ml of MIIYE medium solidified with 2% (w/v) agar in shallow enamel trays (35x25cm) was inoculated with 25ml of an overnight bacterial culture and incubated at 30°C for 4 days. Growth was harvested from the surface using a clean glass slide, suspended in 0.85% (w/v) saline containing 1% (v/v) formalin and stirred in a blender for 1 min. at room temperature. Cells were removed from the preparation by centrifugation (44000g; 30 min.), and the supernate was poured into 3 volumes of cold acetone to precipitate the exopolysaccharide.

### 2.11.2 Purification

The precipitated exopolysacharide was resuspended in distilled water and dialyzed against running tap water for 48h and distilled water for 24h. The preparation was then ultracentrifuged for 45 min. (120000g; MSE Prepspin 65, Sussex, England) and the supernate reprecipitated in acetone, resuspended in distilled water and dialyzed as before. Further purification was achieved by conversion to the sodium form using a cation exchange resin (BDH Amberlite resin IR-120 (Na)) and to the chloride form using an anion exchange resin (BDH Amberlite resin IRA-400 (Cl)). The purified exopolysaccharide was then frozen and lyophilized.

## 2.12 Analysis of Exopolysaccharides

### 2.12.1 Acid Hydrolysis

400µl of 1M Trifluoroacetic acid was added to 2mg of lyophilized polysaccharide preparations in glass ampoules, which were subsequently sealed. Hydrolysis was achieved by heating at 100°C for 16h. The hydrolysates were evaporated to dryness under reduced pressure, washed with distilled water and evaporated (x2) and finally resuspended in 50µl distilled water.

### 2.12.2 Paper Chromatography

Whatman no.1 paper was used for descending paper chromatograpy for 30h in either butan-1-ol/pyridine/water (6:4:3) or ethyl acetate/acetic acid/ formic acid/ water (18:3:1:4). Acid hydrolysates were applied in 5µl aliquots and 2µl aliquots of 0.1M sugar solutions were run as standards. Chromatograms were developed by the alkaline silver nitrate method (Trevelyan et al., 1950).

### 2.12.3 High Performance Liquid Chromatography

Polysaccharide samples were hydrolysed with 1N $H_2SO_4$ at 100°C for 18h and neutralized with BDH Amberlite resin IR-410 ($CO_3^-$). The hydrolysates were then filtered, washed (x2) with distilled water and evaporated under reduced pressure to dryness.

Resuspension of the samples in 125 µl distilled water was followed by filtration (HV 0.45µm filters, Millipore, Japan).

Samples were then directly injected into a Nucleosil (5µm) $NH_2$ column with acetonitrile and distilled water (75:25 v/v) as eluent. Elution was monitored using a Knauer refractometer. Peaks were calibrated according to peak height.

2.12.4 Gelling Properties

500µl aliquots of the appropriate concentrations of exopolysaccharide solutions were added to 500µl aliquots of 1% (w/v) solutions of the chloride salts of a number of cations, and mixed by vortexing. Solutions were observed after 2h and overnight at room temperature for interactions resulting in gelling, precipitation or flocculation.

2.12.5 Viscometry

The viscosities of 2ml aliquots of exopolysaccharide solutions at the appropriate concentrations were measured using a Brookfield Digital cone plate viscometer between shear rates of 0.6 and 120 $sec^{-1}$. A temperature of 25°C was maintained throughout.

2.12.6 Analytical Assay Procedures

All analyses were performed in triplicate and were micromodifications of original assay procedures. Total carbohydrate

content was determined by the phenol-sulphuric method of Dubois et al. (1956), and protein by the method of Lowry et al. (1951). Acetate, pyruvate and uronic acid components were determined by the hydroxamic acid (Hestrin, 1949), dinitrophenylhydrazine (Sloneker & Orentas, 1962) and meta-hydroxydiphenyl (Blumenkrantz & Absoe-Hansen, 1973) methods respectively.

## 2.13 Isolation of Outer Membranes

4x1L volumes of fresh medium were inoculated with 100ml aliquots of an overnight culture (10% v/v inoculum) and incubated until an $E_{600}$ of 0.8 units was reached. The logarithmic phase cells were collected by centrifugation (24000g; 30 min.), resuspended in 20ml 15% (w/v) glycerol and frozen at -80°C.

Two methods were used for outer membrane separation. All steps were performed on ice or at 4°C unless otherwise stated.

### 2.13.1 Osborn Method

The general procedure of Osborn et al. (1972) was followed: Cells collected as above were resuspended in 100ml of cold 0.75M sucrose, 10mM Tris-HCl buffer, pH 7.8. Lysozyme (5ml of 2mg/ml solution) was immediately added and the sample was incubated on ice for 2 min. 2 vols. of cold 1.5mM EDTA (sodium salt), pH 7.5 were added slowly under the cell suspension surface over a 15 min. period during which the suspension was constantly swirled. The sample was incubated on ice until sphaeroplasting was observed by phase

contrast microscopy. Unlysed cells and debris were removed by centrifugation (7000g; 15 min.), the sphaeroplasts collected from the supernate by ultracentrifugation (50000g; 45 min.) and resuspended in 20ml of distilled water. The suspension was sonicated in a series of 30 sec. exposures at a setting of 7 microns in a MSE 100W Ultrasonicator, until the absorbance of the suspension decreased to approximately 20% of its original value. Membranes were collected from the suspension by centrifugation (100000g; 45 min.) and washed in distilled water. Inner and outer membranes were separated as follows. The membranous material was resuspended in 10ml of 1% (w/v) sarkosyl and incubated at room temperature for 20 min. in a shaking water bath. This procedure solubilized the inner membrane, thus the outer membrane could be collected by centrifugation (100000g; 45 min.). The outer membrane was then washed in distilled water, frozen and lyophilized.

2.13.2 PBS-sarkosyl Method

Cells collected as above were washed and respun (44000g, 30 min.) in phosphate-buffered saline (PBS, Table 2.6), then resuspended in 20ml PBS. Cell breakage was achieved by three passages of the suspension through a French pressure cell (Aminco, Silver Spring, M.D., USA). Unbroken cells and debris were removed by centrifugation (7000g, 15 min.) and the membranous material collected from the supernate by centrifugation (50000g, 45 min.). The soluble fraction was removed by pipette and retained. The pellet was resuspended in 1ml of 20% (w/v) sarkosyl and incubated at

Table 2.6: Phosphate-Buffered Saline (PBS)

| | |
|---|---|
| NaCl | 8.00g |
| $Na_2HPO_4$ | 1.15g |
| KCl | 0.20g |
| $KH_2PO_4$ | 0.20g |
| Distilled water | 1l |
| pH | 7.2 |

Table 2.7: Sample Buffer

Final concentrations: 0.01M Tris-HCl (pH 6.8)

1% SDS

0.1% B-mercaptoethanol

10% glycerol

0.005% bromophenol blue

room temperature for 20 min. to solubilize the inner membrane. The outer membrane was collected by centrifugation (140000g, 45 min.), washed in PBS, frozen and lyophilized.

## 2.14 Isolation of Soluble Cell Free Extracts

The soluble fraction retained during the PBS-sarkosyl method (above) was dialyzed against polyethylene glycol to reduce the volume, and subsequently dialyzed agaist distilled water for 24h, frozen and lyophilized.

## 2.15 Polyacrylamide Gel ELectrophoresis

### 2.15.1 Apparatus

Apparatus for polyacrylamide gel electrophoresis was supplied by Raven Scientific Ltd. (Haverhill, Suffolk). Gels were formed between chromic acid cleaned glass plates (18x16x0.1cm) placed together with perspex spacers and sealed with soft paraffin wax.

### 2.15.2 Preparation of Polyacrylamide Gels

A sodium dodecyl sulphate (SDS)-discontinous buffer system was employed with linear gradient gels (7-15% acrylamide). 30% stock acrylamide was prepared by dissolving 29.2g of acrylamide and 0.8g of bis-acrylamide in a total volume of 100ml distilled water. The solution was filtered through glass wool and stored at $4^{o}C$ in a dark

bottle.

The linear gradient resolving gels were prepared using the appropriate volumes of stock acrylamide, 1.5M Tris-HCl (pH 8.8), 0.4% SDS (0.25 vol.) and distilled water. Gel mixtures were degassed, the ammonium persulphate catalyst was added, 0.0019 vol. and 0.0028 vol. of a 10% (w/v) solution for the low and high acrylamide concentrations respectively. This was followed by the addition of accelerator, N,N,N´,N´-tetramethylethylene diamine (TEMED), 0.00125 vol. to both gel mixtures. The gradient gels were poured using a perspex gradient mixer and peristaltic pump. Water-saturated butyl alcohol was overlayered to exclude oxygen from the mixture and allow polymerization.

Stacking gels comprised 0.16 vol. stock acrylamide, 0.25 vol. 0.5M Tris-HCl (pH 6.8), 0.4% SDS and 0.59 vol. distilled water. 0.0028 vol. catalyst and 0.0016 vol. accelerator were added after degassing.

### 2.15.3 Sample Preparation and Electrophoresis

Outer membrane and soluble material prepared as described above were suspended in distilled water and an equal volume of double strength sample buffer (Table 2.7). Samples were heated to 100$^{o}$C for 10 min., allowed to cool and loaded onto gels in the appropriate volumes (20-100µl). A range of protein standards containing 1µg/ul were prepared by the same procedure and 25µl volumes applied to the gels.

Gels were initially run at a constant current of 15mA until the

dye reached the resolving gel and subsequently at 35mA until the dye front reached 1cm from the base of the gel. Running buffer used is described in Table 2.8.

### 2.15.4 Analysis of Gels

Protein staining was either by PAGE blue 83 with subsequent destaining or by silver stain (Biorad labs, bulletin 1089).

The Rf of protein standards was calculated by multiplying the distance of protein migration by the length of the gel before staining and dividing the figure obtained by the length of the gel after staining and the distance of dye migration. This takes account of the gels swelling during the staining procedure.

Standard curves were prepared by plotting $\log_{10}$ molecular weight against Rf value and the molecular weights of the sample proteins estimated.

Results were recorded by photography (Minolta X-500, 35mm camera ) and drying of gels (Zabona gel drier, Basle).

## 2.16 Immobilized Metal Affinity Chromatography

Outer membranes were analyzed for specific copper-binding proteins by application to chelating Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden). The gel was packed in a column, charged by washing with a solution of $CuSO_4 . 5H_2O$ (5mg/ml) and equilibrated with 5 column volumes of 0.05M phosphate buffer, pH 7.5 (Cruickshank, 1965) containing 0.5M NaCl (starting buffer).

Table 2.8: Running Buffer

| | |
|---|---|
| Tris-base | 3.0g |
| Glycine | 14.4g |
| SDS | 1.0g |
| Distilled water | 1l |

Table 2.9: Page Blue 83 Stain

| | | |
|---|---|---|
| Stain: | Page Blue 83 | 1.25g |
| | Methanol | 227ml |
| | Glacial Acetic Acid | 46ml |
| | Distilled water | to 500ml |

Filtered

Staining process for a minimum of 4h, or overnight

| | | |
|---|---|---|
| Destain: | Methanol | 50ml |
| | Glacial Acetic Acid | 75ml |
| | Distilled water | to 1l |

Changed regularly until background cleared

Outer membranes were solubilized by heating to 100°C for 10 min. in 1ml starting buffer containing 1% Tween 80. Samples were applied at a flow rate of approximately 2ml $cm^{-2}h^{-1}$ and unbound material washed through with starting buffer. Specifically bound protein was recovered by a gradient reduction in pH using starting buffer (pH 7.5) and 0.05M Tris-acetate (pH 3.0), 0.5M NaCl. The column was subsequently regenerated by stripping off the chelated metal using 2 column volumes of 0.05M EDTA, 1M NaCl, and washing with starting buffer. All buffers contained 0.02% (w/v) sodium azide.

Material was eluted in 1ml fractions and protein was monitored by absorbance at 280nm. Bound and unbound material was reduced in volume by dialysis against polyethylene glycol, redialyzed in distilled water for 24h, frozen and lyophilized and subsequently analyzed by SDS-PAGE.

## 2.17 Fine Chemicals and Biochemicals

All chemicals and biochemicals were purchased from BDH Chemicals Ltd., Poole, England, or the Sigma Chemical Company Ltd., Kingston upon Thames, England unless otherwise stated.

Dimethyltin dichloride (dichloromethylstannane) was obtained from Fluka A.G., Buchs, Switzerland).

# CHAPTER THREE: RESULTS & DISCUSSION

## 3.1 Isolation and Characterization of Marine Bacteria

Generalizations on the attachment of marine bacteria to surfaces are difficult to make due to differences in the range of bacterial species used in individual studies. Bacterial strains differ considerably in the quality and quantity of cell surface polymers, thus it is not surprising that they also vary in attachment ability and strength (Fletcher, 1980). Although studies have been performed to determine the bacterial types involved in the successional development of microbial biofilms (Dempsey, 1981a,b; Marshall et al., 1971b), little attempt has been made to characterize adherent marine bacteria or to determine the relationship between adherent bacteria and the total marine population.

### 3.1.1 Isolation of Bacteria

The range of bacteria isolated from the marine environment included both adherent and free-living species (Table 3.1). A wide variety of colony types was found on natural and glass surfaces and in seawater, but the diversity of attached bacteria was reduced in the presence of antifouling paints. This may indicate that relatively few strains of adherent bacteria are able to tolerate the toxicants used in these paints (Dempsey, 1981a,b).

Table 3.1: Morphological and Physiological Characterization of Marine Isolates

| Isolate | Source | Capsule/slime[a] | Glycogen[b] | Haemagglutination |
|---|---|---|---|---|
| 82A | Natural marine surfaces | c/s | +/- | - |
| 82B | | c | +/- | - |
| 82C | | c | - | - |
| 82D | | c | - | - |
| 82F | | c | +/- | - |
| 82G | | c | - | - |
| 82H | | c | - | - |
| 82I | | c | - | - |
| 82J | | c | - | - |
| 82L | | s | - | - |

Table 3.1: Morphological and Physiological Characterization of Marine Isolates (contd.)

| Isolate | Source | Capsule/slime[a] | Glycogen[b] | Haemagglutination |
|---|---|---|---|---|
| 82M | Seawater | c | +/- | - |
| 82O | | c | - | - |
| 82P | | c | - | - |
| 82Q | Glass surfaces | s | - | - |
| 82S | | s | - | - |
| 82T | | - | - | + |
| 82U | | c | - | - |
| 82V | | c/s | - | - |
| S3/A51 | S3 painted surface | nd | nd | nd |

a: c - capsule; s - slime

b: - - no glycogen production; +/- - indistinct reaction

nd - not done

## 3.1.2 Characterization of Bacteria

### 3.1.2.1 Morphological and Physiological

All isolates were found to be Gram-negative, motile rods or coccobacilli. Few produced glycogen inclusion granules, but the majority produced extracellular capsules or slime (Table 3.1). The one exception was isolate 82T, which was the only strain to show haemagglutination of horse red blood cells. Haemagglutination by bacterial cells is an indication that they may possess fimbriae, which have been implicated as organelles of attachment (Duguid, 1959). This may reflect a difference in the mechanisms of irreversible attachment between isolate 82T and the exopolysaccharide-producing strains.

The bacterial isolates exhibited differences in their abilities to grow over a temperature range from 7°C to 37°C (Table 3.2). However all strains had an optimum growth temperature of 30° C, which was selected for the routine growth of cells.

Isolate S3/A51 exhibited distinct staining properties with the Gram procedure. Irregular Gram-negative rods were partly replaced in older cultures by Gram-variable forms, either cocci or elongated rods (not shown). V-forms of rod-shaped cells were also observed. These morphological features are similar to those, described by Clark (1972) and Duxbury & Gray (1977), of the morphogenetic cycle of Arthrobacter species, and may indicate that isolate S3/A51 is a marine Arthrobacter.

The morphological and physiological characteristics of the

Table 3.2: Range of Growth Temperatures

| Isolate | 7°C | 20°C | 30 °C | 37°C |
|---|---|---|---|---|
| 82A | + | + | + | - |
| 82B | + | + | + | + |
| 82C | +/- | +/- | + | + |
| 82D | + | +/- | + | + |
| 82F | + | + | + | + |
| 82G | + | + | + | + |
| 82H | +/- | +/- | + | - |
| 82I | + | + | + | +/- |
| 82J | + | + | + | + |
| 82L | + | + | + | + |
| 82M | +/- | +/- | +/- | +/- |
| 82O | + | + | + | +/- |
| 82P | + | + | + | +/- |
| 82Q | + | + | + | + |
| 82S | +/- | +/- | + | + |
| 82T | +/- | +/- | + | +/- |
| 82U | +/- | - | +/- | +/- |
| 82V | + | +/- | + | +/- |
| S3/A51 | + | + | + | + |

bacterial isolates do not reveal any distinct differences between adherent and non-adherent bacteria. However, antifouling paint-coated surfaces may select for a specific range of bacteria, as isolate S3/A5l showed different properties.

3.1.2.2 Biochemical

Isolates showed a wide range in ability to utilize citrate; hydrolyse starch, gelatin and Tween 80; and to produce oxidase, urease and hydrogen sulphide. Catalase production occurred in all strains (Table 3.3). The majority of the isolates had negative reactions in fermentation tests with a number of sugars (Table 3.4). This indicates an oxidative breakdown of carbohydrates. A few isolates (82A, 82B, 82J, 82L, 82M and 82V) produce acid, or acid and gas, from some or all of the sugars tested. Again no particular pattern emerged to differentiate between bacteria isolated from surfaces and those isolated from seawater.

Corpe (1970b) identified a bacterium as Pseudomonas atlantica on the basis of its morphological, physiological and biochemical characteristics. This species was described as being representative of a number of bacterial species in the marine environment that attach to surfaces. Pseudomonads are Gram-negative rods, are catalase positive, are usually oxidase positive, and breakdown carbohydrates oxidatively (Palleroni, 1984). Some marine pseudomonads have been found to be oxidase negative, for example Pseudomonas marina. Thus it is possible that several of these isolates could be marine pseudomonads.

Table 3.3: Biochemical Characterization of Marine Isolates

| Isolate | a | b | c | d | e | f | g | h |
|---|---|---|---|---|---|---|---|---|
| 82A | - | + | + | + | + | - | - | - |
| 82B | - | - | - | + | + | + | - | + |
| 82C | - | + | + | + | + | + | + | + |
| 82D | - | + | + | + | + | - | - | - |
| 82F | - | + | + | - | + | - | - | - |
| 82G | + | + | + | + | + | - | - | - |
| 82H | - | + | - | + | + | + | + | + |
| 82I | + | + | + | + | + | - | - | - |
| 82J | - | - | - | + | + | + | - | - |
| 82L | - | - | - | - | + | + | + | + |
| 82M | - | - | - | - | + | - | - | - |
| 82O | - | + | + | + | + | + | - | - |
| 82P | + | + | + | + | + | - | - | - |
| 82Q | + | + | + | + | + | - | - | - |
| 82S | - | + | + | + | + | - | - | - |
| 82T | + | - | - | - | + | - | - | - |
| 82U | - | - | - | - | + | - | - | - |
| 82V | - | - | - | - | + | - | + | + |

a- hydrolysis of starch; b- hydrolysis of gelatin; c- hydrolysis of Tween 80; d- oxidase production; e- catalase production; f- urease production; g- $H_2S$ production; h- citrate utilization.

Table 3.4: Sugar Fermentations

| Isolate | Dex | Suc | Lac | Glc | Dul | Manl | Fru | Mal |
|---|---|---|---|---|---|---|---|---|
| 82A | A | - | - | - | - | - | - | - |
| 82B | A | - | - | A/G | - | A/G | A/G | A/G |
| 82C | - | - | - | - | - | - | - | - |
| 82D | - | - | - | - | - | - | - | - |
| 82F | - | - | - | - | - | - | - | - |
| 82G | - | - | - | - | - | - | - | - |
| 82H | - | - | - | - | - | - | - | - |
| 82I | - | - | - | - | - | - | - | - |
| 82J | A/G | A/G | A/G | - | - | - | - | - |
| 82L | A/G | A/G | A/G | A/G | A/G | A/G | A/G | A/G |
| 82M | A/G | A/G | A/G | - | - | - | - | - |
| 82O | - | - | - | - | - | - | - | - |
| 82P | - | - | - | - | - | - | - | - |
| 82Q | - | - | - | - | - | - | - | - |
| 82S | - | - | - | - | - | - | - | - |
| 82T | - | - | - | - | - | - | - | - |
| 82U | - | - | - | - | - | - | - | - |
| 82V | A | - | - | A | - | A | A | A |

-: no reaction

A: acid production only

A/G: acid and gas production

Isolates that are oxidase negative and breakdown carbohydrates by fermentation (82L, 82M, 82V) may be members of the class Enterobacteriaceae.

A wider range of tests might allow more positive identification of these isolates. Zambon et al. (1984) classified adherent bacteria isolated from glass surfaces by biochemical tests, flagellar arrangement and the API 20E system, which utilizes stored computer data on profiles of biochemical patterns found in the Gram-negative bacteria for identification. Pseudomonads were found to be predominant. Immunofluorescent antibodies to the predominant organisms were prepared and enabled the in situ identification of these species in microfouling films. With the development of a greater number of specific immunological reagents, this technique may provide a rapid and reliable means of bacterial identification in microbial films.

### 3.1.3 Extracellular Polysaccharides Produced by Marine Isolates

Extracellular polysaccharides from the marine isolates were examined by hydrolysis and paper chromatography. All appeared to contain glucose and a uronic acid, and all except that of isolate 82D contained galactose. Other components varied, and included mannose, rhamnose and either fucose or N-acetyl glucosamine (Table 3.5). It was not possible to distinguish between fucose and N-acetyl glucosamine with the solvent system used. However it would be expected that hydrolysis would remove some of the N-acetyl groups from N-acetyl glucosamine, resulting in the appearance of two

Table 3.5: Sugar Constituents of Extracellular Polysaccharides Produced by Marine Isolates

| Isolate | Glc | Gal | Man | Rha | Ur A* | Fuc |
|---|---|---|---|---|---|---|
| 82A | + | + | - | + | + | + |
| 82B | + | + | - | + | + | - |
| 82C | + | + | - | + | + | - |
| 82D | + | - | - | - | + | - |
| 82F | + | + | - | + | + | + |
| 82G | + | + | +/- | +/- | + | + |
| 82H | + | + | - | + | + | + |
| 82I | + | + | +/- | +/- | + | + |
| 82J | + | + | - | + | + | + |
| 82L | + | + | + | - | + | - |
| 82M | + | + | + | +/- | + | - |
| 82O | + | + | - | + | + | + |
| 82P | + | + | - | - | + | + |
| 82Q | + | + | - | + | + | + |
| 82S | + | + | - | + | + | + |
| 82T | + | + | - | + | + | + |
| 82U | + | + | + | + | + | + |
| 82V | + | + | - | - | + | + |
| S3/A51 | + | + | - | - | + | + |

*: Uronic acid

spots on the chromatogram corresponding to N-acetyl glucosamine and glucosamine respectively. This was not observed, thus this component is more likely to be fucose. There was no indication from these results that any distinct chemical type of polysaccharide is produced by adherent bacteria.

Although the association of polysaccharide with microbial attachment in aqueous environments has been demonstrated (Allison & Sutherland, 1984; Corpe, 1970b), few of the polymers found in these systems have been analyzed. Corpe (1970b) reported that a polysaccharide from Pseudomonas atlantica contained mannose, glucose, galactose and galacturonic acid. These monosaccharides were also found to occur most frequently among polymers from a range of freshwater and marine bacteria (Sutherland, 1980). However, Uhlinger & White (1983) indicate that, in addition to these components, a polysaccharide from P. atlantica contained rhamnose, fucose, arabinose,xylose, mannuronic acid and glucuronic acid, and that the composition of the polymer varied during the growth cycle.

It is clear that polysaccharides that may be involved in the attachment process can be of various chemical types. This would indicate that the secondary or tertiary structure of a polysaccharide is more likely to play a significant role in attachment than is the primary chemical structure (Sutherland, 1982). In addition, if more than one polysaccharide is involved, the possibility of co-gellation or other forms of interaction should be considered. The interaction of ions with exopolysaccharide may cause formation of gels or flocs, which could be important in their role in adhesion (Sutherland, 1980). Further analysis of adherent

polysaccharides is required to determine if adherent properties are related to any particular structural type.

It should be noted that, in studies on the role of bacterial polysaccharides in the attachment process, difficulties exist in the extrapolation of laboratory results to the natural environment. Polysaccharides produced under laboratory conditions might differ from those produced by the same species in the natural environment. In addition, it is difficult to demonstrate the definite involvement of the polysaccharide in the attachment process. However, these studies allow some determination of the characteristics of bacterial polysaccharides which may be involved in attachment in marine systems.

### 3.1.4 Isolation of Copper-resistant Mutants

Naturally occurring, copper-resistant mutants of isolate 82Q were selected by replica plating (Table 3.6). Single colonies of mutant 82Q/15b transferred from plates containing 1mM copper sulphate to SWYE plates, and subsequently replica plated onto copper containing plates, retained their ability to tolerate divalent copper ions. This was also observed with isolate S3/A51 (Plate 1), and indicated that copper resistance in these strains is stably inherited. Thus, this copper resistance may be chromosomally-encoded. However, other authors report the involvement of an inducible plasmid in copper tolerance in *E. coli* (Rouch *et al.*, 1985; Tetaz & Luke, 1983).

Table 3.6: Copper-Resistant Mutants

| Mutant | Tolerable Copper Concentration (mM) |
|---|---|
| 82Q (Parental strain) | 0.4 |
| 82Q/1a | 0.5 |
| 82Q/12b | 0.5 |
| 82Q/15a | 0.8 |
| 82Q/15b | 1.0 |

Plate 1: Single colonies of mutant 82Q/15b (a) and isolate S3/A51 (b) growing on plates containing 1mM copper sulphate were transferred to master plates containing no added toxicant (left). Subsequent replica plating onto plates containing 1mM copper sulphate (right) showed that both strains retained their ability to tolerate copper ions.

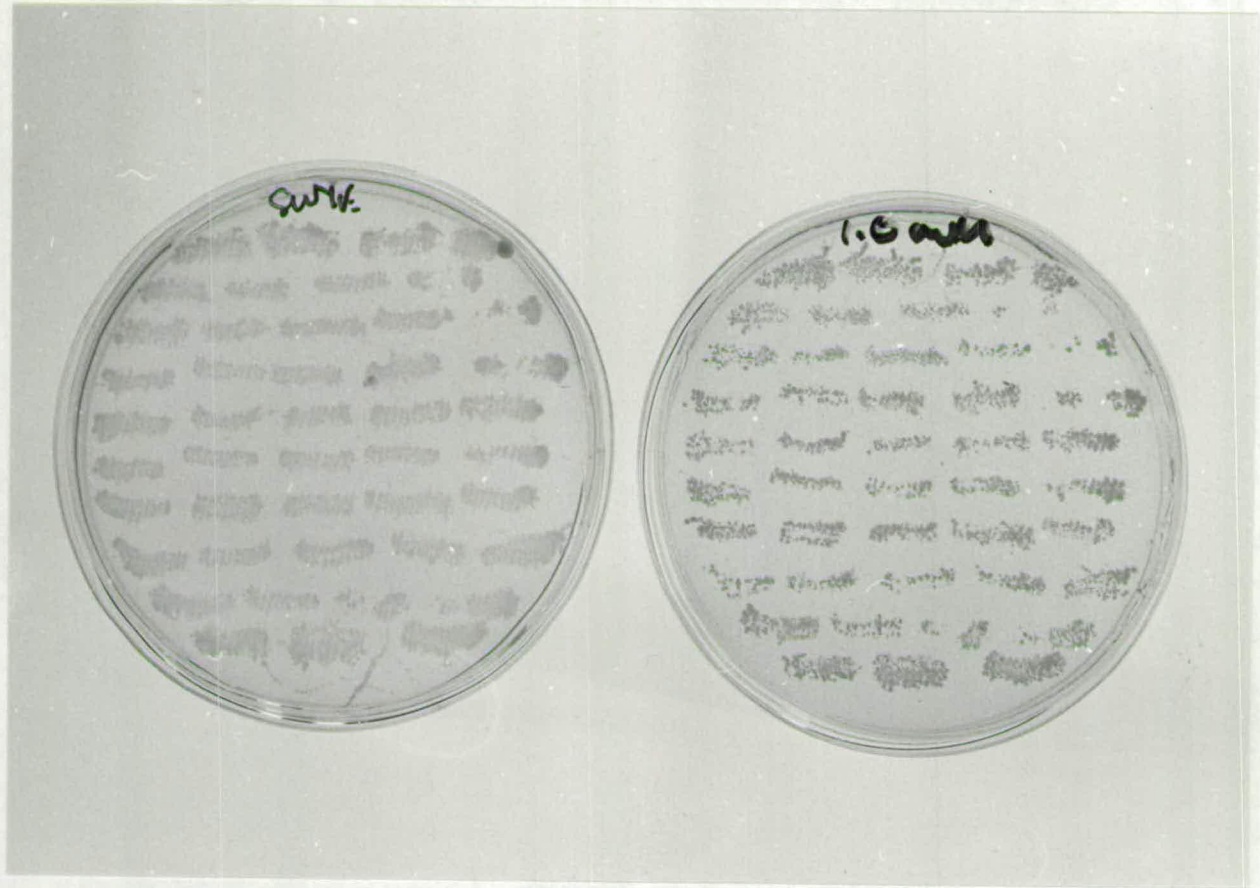


Plate 1(a)

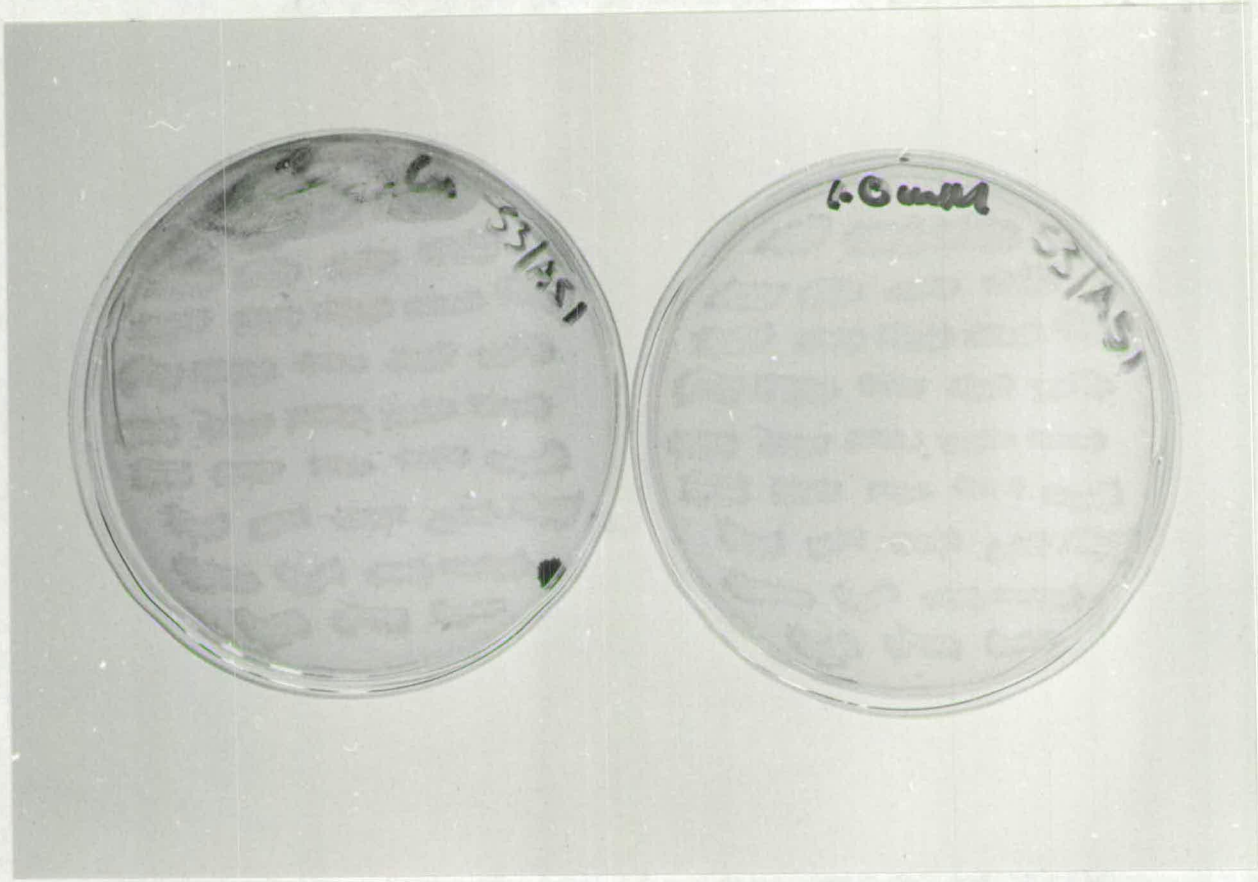


Plate 1 (b)

## 3.2 Growth and Morphological Changes of Marine Bacteria in the Presence of Toxicants

The toxicity of heavy metals and organometallic compounds to microorganisms is influenced by many factors (see Section 1.8.4). Hallas & Cooney (1981) report that the chemical form of tin is important when assessing its toxicity, as estuarine microbial populations were found to be more sensitive to organotin than to inorganic tin. The chemical form of copper also has an influence on its toxicity. For example, the cuprous chloride ion is more toxic to marine bacteria than the cupric ion (Dempsey, 1983).

The concentration of a toxicant introduced into the environment will influence the response of the microbial population to it. Low concentrations of a toxicant may stimulate the growth and metabolic activities of microorganisms, as discussed by Babich & Stotzky (1980). The accumulation of a non-lethal concentration of a toxicant at cell surfaces may induce an alteration in permeability, allowing a freer flow of nutrients across the plasma membrane and, thereby, an increase in cellular metabolic activity. Furthermore, some chemicals that can be toxicants are required nutrients for microbial growth in trace amounts. Methanol-utilizing bacteria have been shown to require copper for growth and methanol utilization (Vrdoljak *et al.*, 1984) and for activity of the enzyme, methane monooxygenase (Prior & Dalton, 1985). However, as the concentration of copper is increased, a toxic effect can be demonstrated, resulting in the inhibition of enzyme activity (Prior & Dalton, 1985).

In addition, the characteristics of the microbial populations in the environment will affect toxicity (Babich & Stotzky, 1980). These factors include pigmentation, slime production, morphological state, nutritional state and genetic adaptability. A wide range of bacterial characteristics has been demonstrated in bacterial species from the natural marine environment (see Section 3.1), thus the effect of toxicants on these organisms may vary.

### 3.2.1 Toxicant Form

In copper-based paints the toxicant is in the form of cuprous oxide. However, as this leaches from the paint it is rapidly oxidised to the cupric form (fig.3.1). The transient cuprous chloride ion has been found to be more toxic than the cupric ion (Dempsey, 1983), but presumably by the time it reaches the cells it will already have been oxidised. Thus the cupric form was selected for use in studies concerning copper-based paints.

The form of toxicant in some organotin based paints is triphenyltin fluoride (Dempsey, 1981a,b). However this compound is hydrophobic and thus insoluble in routine growth media. Dimethyltin dichloride (DMTC) is a water-soluble organotin compound, which made it suitable for use in studies investigating the effect of organotin-based paints.

The influence of chemical form on the toxicity of tin was examined. Isolate 82Q was grown overnight in media containing a range of concentrations of stannous chloride, stannic chloride or DMTC (fig.3.2). The organotin compound showed the greatest

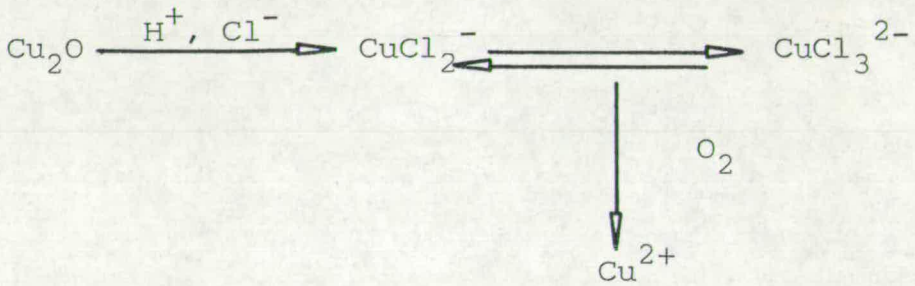
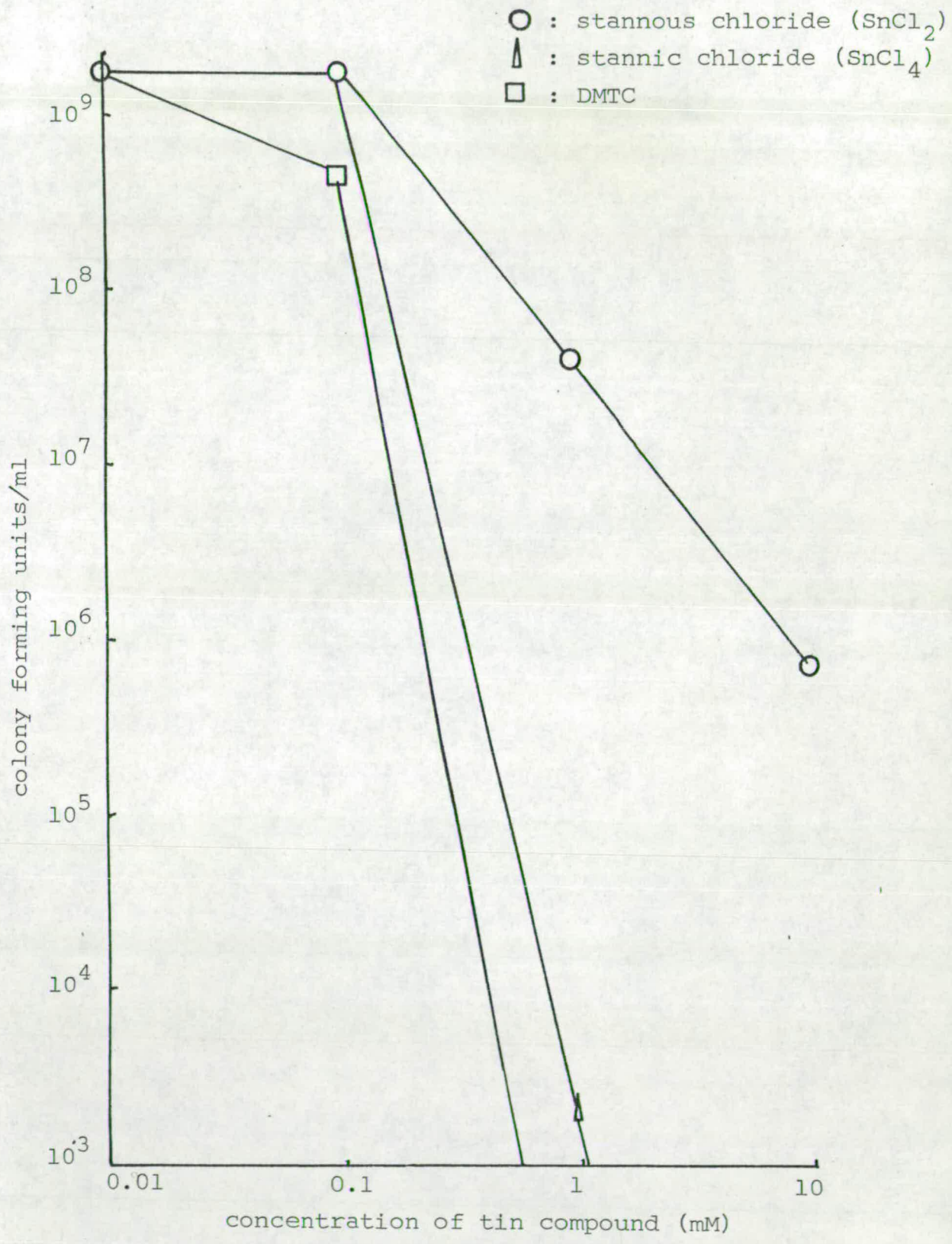



fig.3.1: Sequence of reactions in the dissolution of cuprous oxide in seawater.

fig.3.2: Growth of isolate 82Q in the presence of tin compounds.



inhibition of growth. This is consistent with the work of Hallas & Cooney (1981) who report that estuarine microbial populations were more sensitive to DMTC than to stannic chloride.

### 3.2.2 Toxicant Concentration

The effect of toxicant concentration on growth of a range of marine isolates was tested. Isolates were inoculated into media containing the appropriate concentration of toxicant and incubated under routine growth conditions for 16h. The five isolates showed differences in their abilities to tolerate DMTC over a range of 0.1-0.5mM (fig.3.3), and copper sulphate over a range of 0.1-1.0mM (fig.3.4). This suggests that within the diverse community of microorganisms found in the natural marine environment there will exist a range of bacteria which are able to tolerate the presence of antifouling paints.

Greatest tolerance to copper was exhibited by isolates 82B and S3/A51, and these were selected for further studies on the mechanisms of bacterial tolerance to copper. Copper-resistant mutants of isolate 82Q varied with respect to growth inhibitory concentrations of copper (fig.3.5). 82Q/15b showed little difference in growth with copper sulphate over the range of 0.1-1.0mM, and was thus used in further studies.

The hydrophobic organotin compounds generally used in antifouling paints have little effect on Gram-negative bacteria (see Section 1.8.2). However, DMTC is a water-soluble compound and will thus be able to penetrate the outer membranes of Gram-negative

fig.3.3: Growth of marine isolates in the presence of organotin.

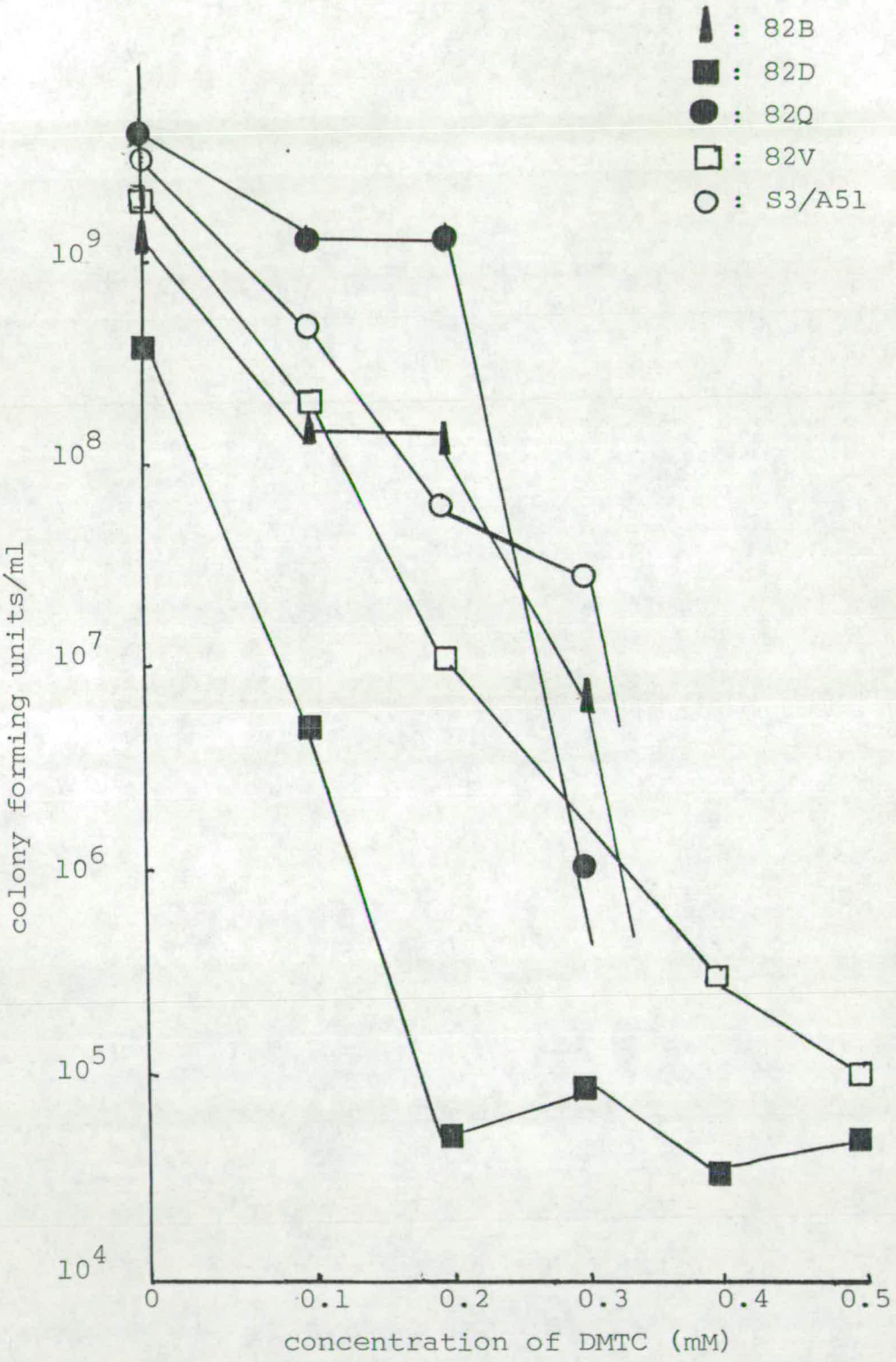


fig.3.4: Growth of marine isolates in the presence of copper sulphate.

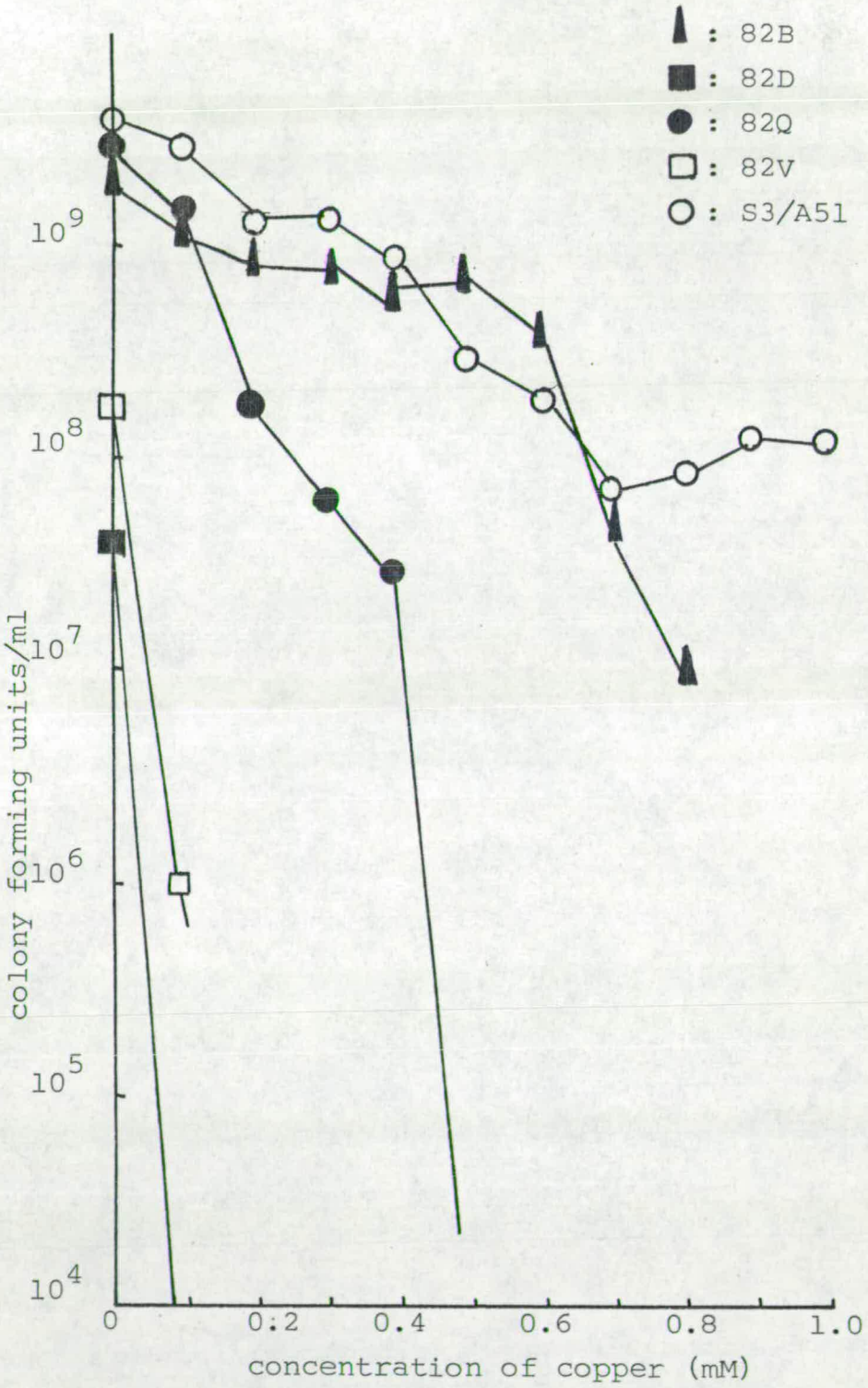
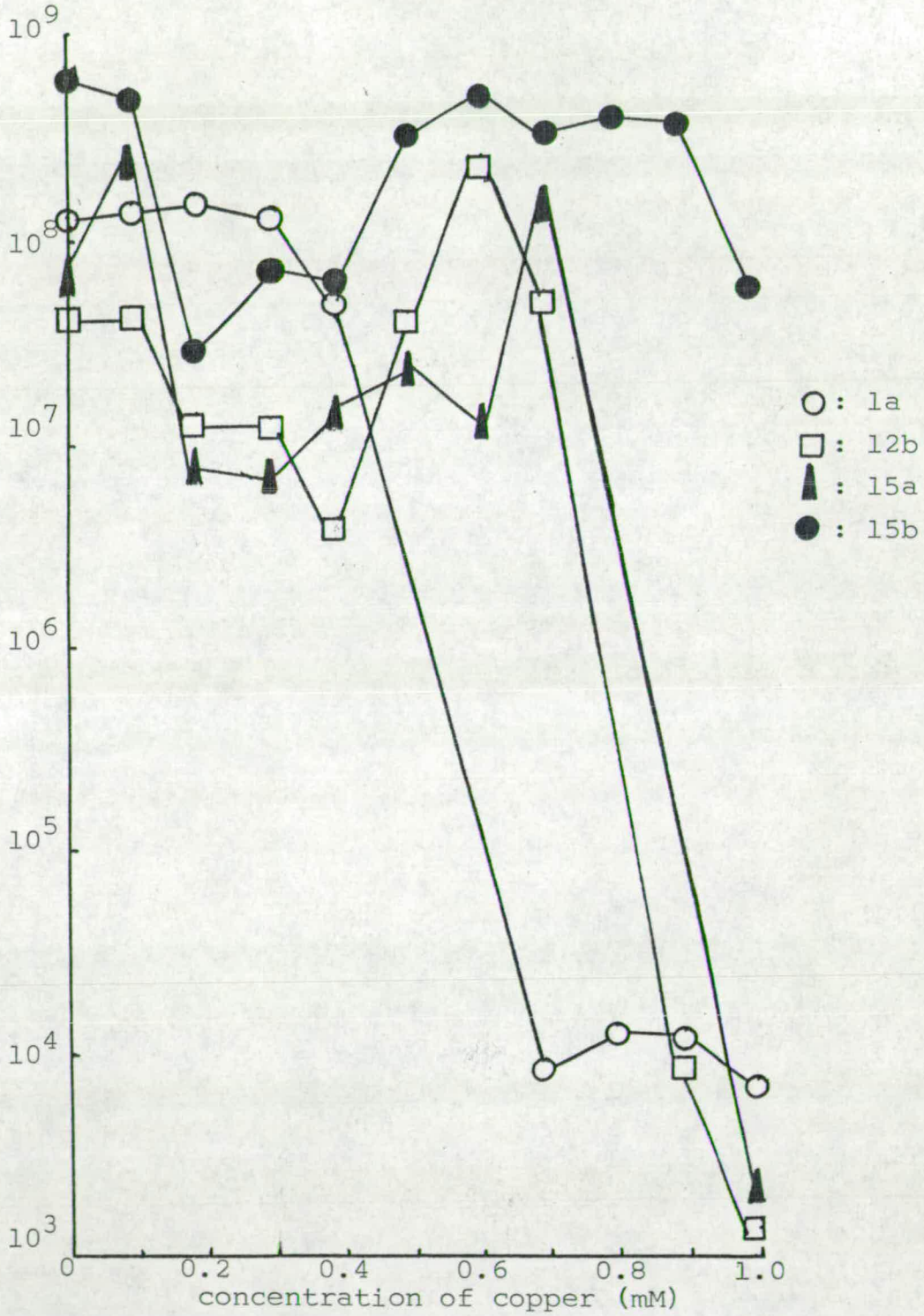


fig.3.5: Growth of mutants of isolate 82Q
in the presence of copper sulphate.



bacteria through the hydrophilic pores formed by major outer membrane proteins. Thus, although DMTC is found to be toxic to these Gram-negative isolates, this effect may not occur with hydrophobic organotin compounds.

Growth media used in these experiments contained both yeast extract and casamino acids. The presence of such compounds has been shown to reduce the toxic effects of copper, presumably due to their ability to chelate metals (Jones, 1964; MacLeod et al., 1967; Ramamoorthy & Kushner, 1975). Thus it must be recognized that the inhibitory concentrations of toxicants demonstrated may be higher than in the absence of soluble organic materials, due to binding and thus reduced availability of the toxicant. However, as the media used in all studies contained the same concentrations of organic components, the relative tolerances of different bacterial isolates will be pertinent.

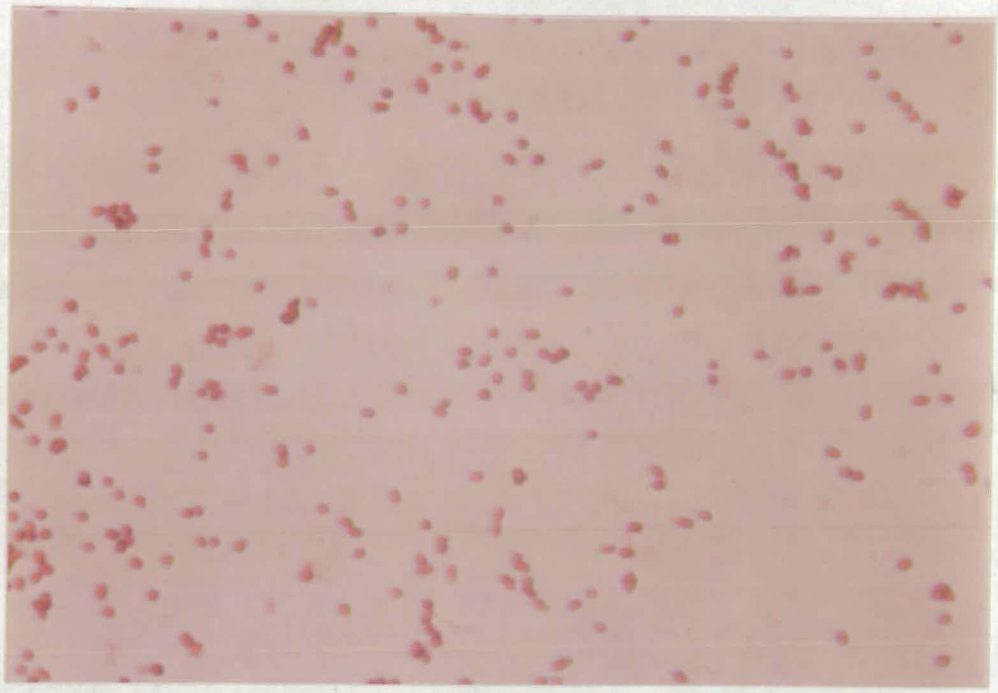
### 3.2.3 Morphological Changes

Isolates 82B and S3/A51 were observed to undergo morphological changes when grown in the presence of relatively high concentrations of copper. Gram stained films of both isolates showed cells of abnormal shape and size (Plate 2). Phase contrast microscopy revealed that these abnormal cells had lost motility and were osmotically stable. Isolate 82Q also exhibited a change in cell shape at concentrations greater than 0.3mM copper. Cells became spherical and lost motility, though no dramatic change in size was seen. No morphological changes were found in any isolate during

Plate 2:

(a) Gram stained films of isolate 82B grown in the absence (i) and presence (ii) of 0.7mM copper sulphate.

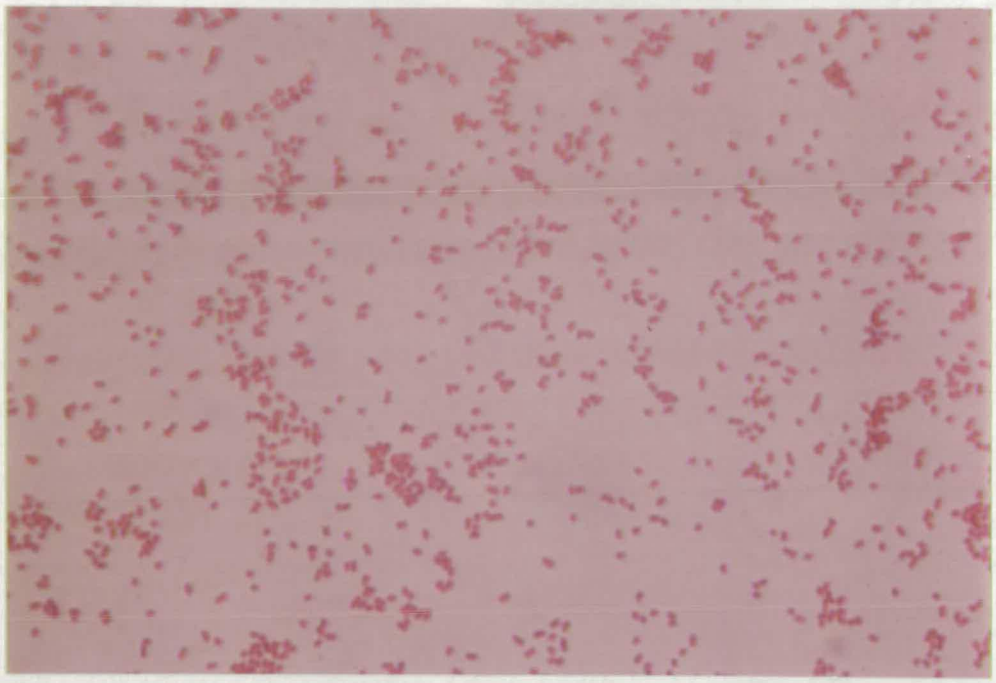
(b) Gram stained films of isolate S3/A5l grown in the absence (i) and presence (ii) of lmM copper sulphate.



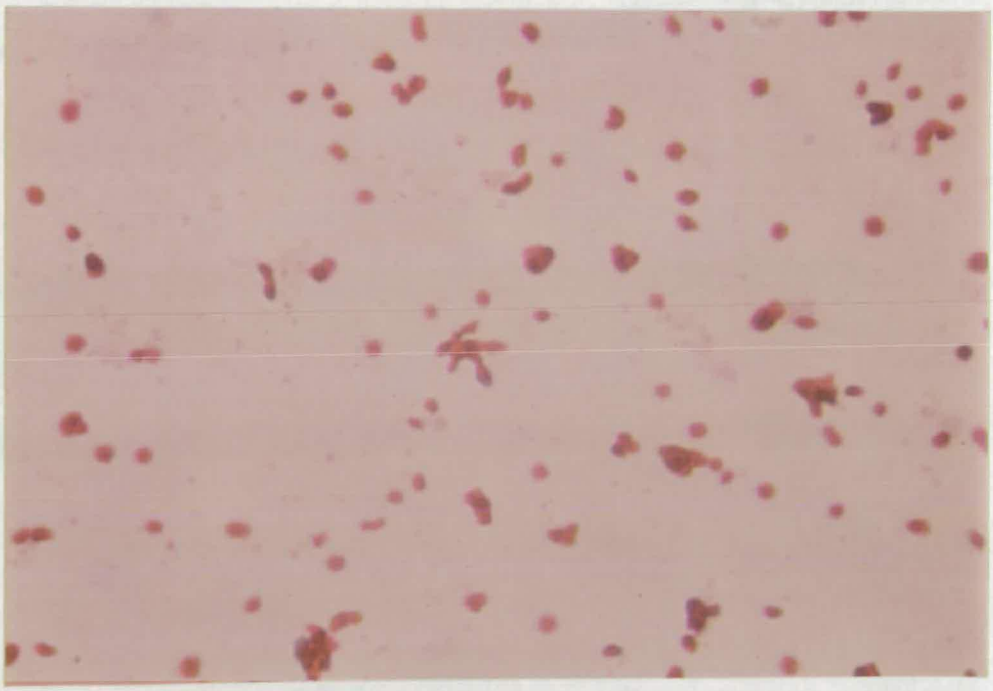
(i)

(ii)

Plate 2 (a)



(i)



(ii)

Plate 2 (b)

growth in the presence of DMTC which indicates a different action on the cell than that of copper.

Scanning electron microscopy of isolate S3/A51 provided further details of the morphological changes (Plate 3). Cells grown in the presence of 1mM copper sulphate showed an increase in size compared to cells grown under routine conditions and appeared to be non-flagellate, which may explain their loss of motility. In addition, there appears to be some effect on cell division or separation mechanisms, as many cells remain linked together. Some cell forms (Plate 3b) are analogous to those described by Duxbury & Gray (1977) in studies on the growth of cystites, cocci and rods of Arthrobacter globiformis. These forms were interpreted by these authors as coccoid cystites with rod-shaped buds produced terminally.

Morphological changes occurring in a marine Arthrobacter grown in the presence of 0.5mM divalent nickel ions were also attributed to an effect on cell division mechanisms (Cobet et al., 1970). Enlarged cells resulted from a continuation of cell growth with cessation of multiplication. However, in this case osmotic instability was observed, the cell wall expanding away from the protoplast to produce the appearance of being plasmolysed.

The change in cell shape in the presence of copper, may suggest interference in the process of cell wall synthesis, as maintenance of shape is dependent on the cell wall, in particular the peptidoglycan layer. This effect shows similarity to the effect of β-lactam antibiotics, such as penicillin, on growing cells (Hammond et al., 1984). When bacteria are exposed to β-lactams, a small

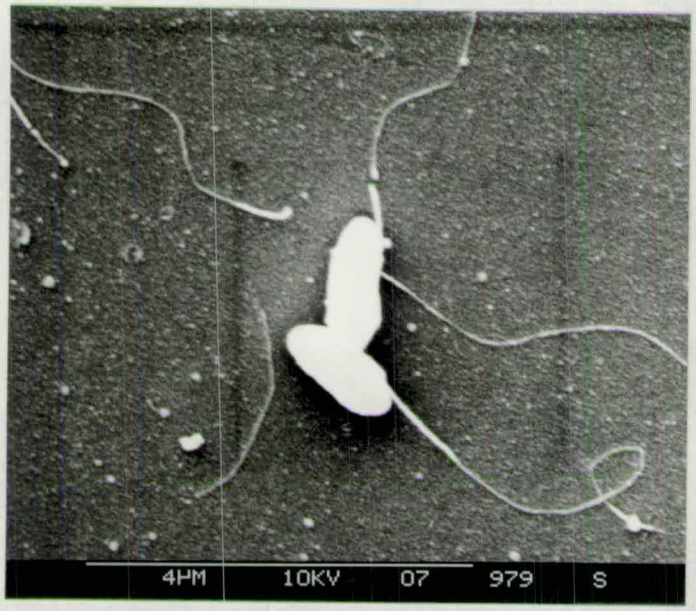
Plate 3: Scanning Electron Micrographs of isolate S3/A5l.

(a) Cells produced during growth under routine conditions.

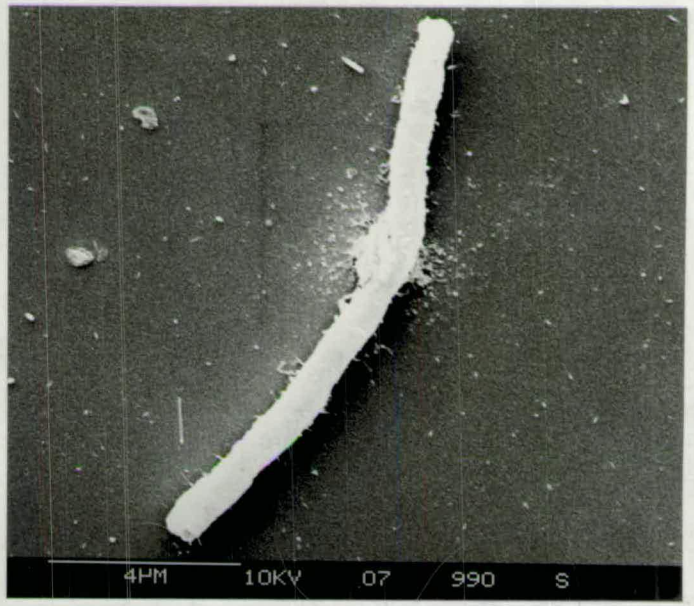
(b) and (c) Cells produced during growth in the presence of lmM copper sulphate.

(d) Microcolony formation by attached cells during growth in the presence of lmM copper sulphate.

(a)


4PM 10KV 07 979 S

(b)


4PM 10KV 07 990 S

(c)


4PM 10KV 07 986 S

(d)


2PM 10KV 07 982 S

Plate 3

amount of the antibiotic becomes covalently linked to the cells. These covalently bound molecules are responsible for damaging and ultimately killing the cell. They are bound to enzymes involved in the synthesis of peptidoglycan, known as penicillin-binding proteins (PBPs), and effectively inhibit their action. Since no other metabolic or biosynthetic activities are affected, the cells continue to grow, but are unable to produce cross-linked peptidoglycan. Thus, abnormal cell shapes develop and the cells eventually stop growing. Lysis is a common result, but filaments and cells with other unusual shapes may persist without lysis.

Thus, the effect of copper on growing cells might be attributed to inhibition of proteins involved in peptidoglycan synthesis (PBPs). This inhibition may result from copper binding to the proteins and rendering them inactive, or by competitively inhibiting the uptake of other metal ions required as cofactors for enzyme activity.

The effect of the toxicants described above will only occur in cells which are actively growing and dividing. Thus, the physiological age of the cells may influence toxicity. Possibly, cells in the stationary phase of the growth cycle, or non-growing cells may show differences in resistance to toxicants.

## 3.3 Effect of Physiological Age of Cells on Tolerance to Toxicants

It has been suggested that the sensitivity of microorganisms to toxicants will be influenced by the physiological age of the cells (Babich & Stotzky, 1980). Cells of different physiological ages may differ in their production of cell surface components. Since the bacterial cell surface is involved in binding and uptake of heavy metals (see Section 1.8.1), this may influence tolerance ability. There is appreciable evidence that production or secretion of bacterial polymers is most common during late logarithmic and stationary phases of growth (Harris & Mitchell, 1973; WIlliams & Wimpenny, 1977). Physiological age of cells has been known to influence their attachment to polystyrene (Fletcher, 1977) and metal (Duddridge et al., 1981) surfaces. This may be explained in terms of the amount and type of extracellular polymeric materials produced at different growth phases. Such polymers could also influence the toxicity of heavy metals to microorganisms.

### 3.3.1 Tolerance to Organotin

Growth curve analyses of isolate 82Q revealed the effect of DMTC when added to cultures of differing physiological age (fig.3.6). Addition of 0.1mM DMTC had little effect on growth of cultures during either lag phase, early logarithmic phase or late logarithmic phase. 1mM DMTC completely inhibited further growth of cultures at any growth phase. Viable cell counts indicated that treatment with 1mM DMTC rendered the cells non-viable at all growth

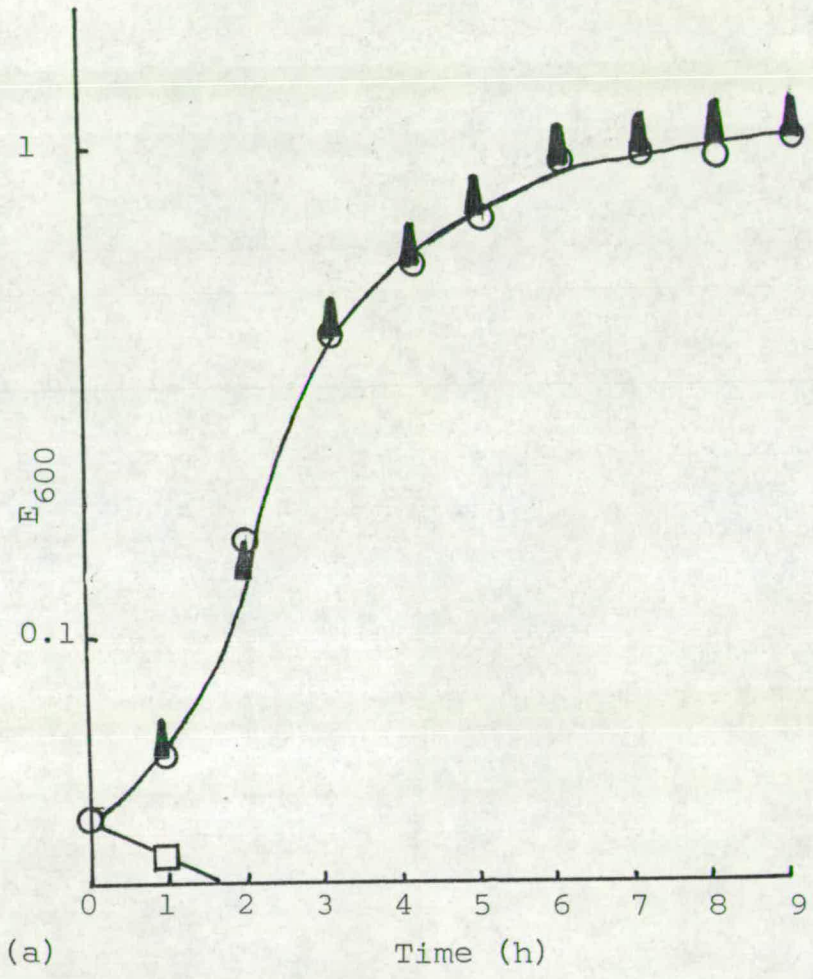
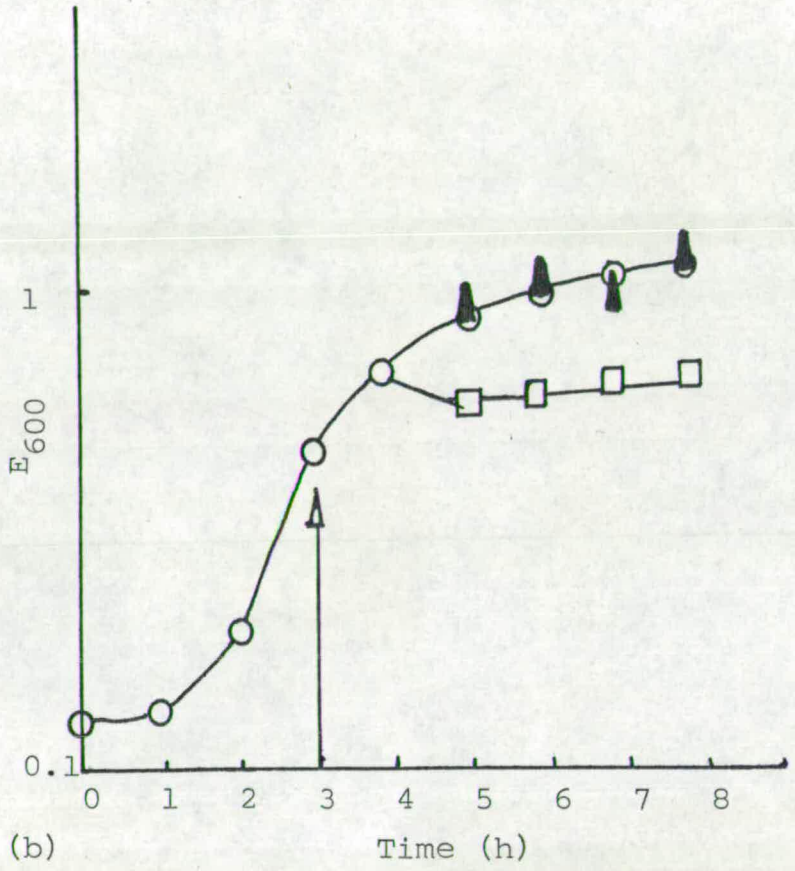
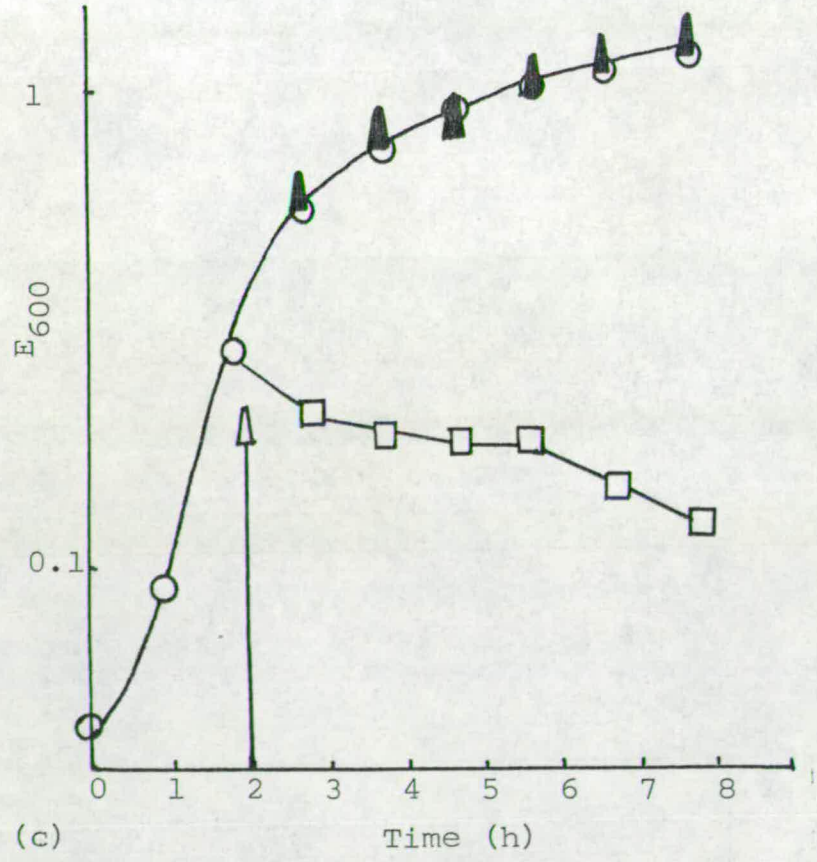



fig.3.6: Effect of DMTC on growth of isolate 82Q when added during lag phase (a), late logarithmic phase (b) and early logarithmic phase (c). Arrow signifies time of DMTC addition.

: no addition of DMTC
: addition of 0.1mM DMTC
: addition of 1.0mM DMTC




(b)




(c)

fig.3.6 (continued)

phases. Thus, with the single species tested here, the physiological age of the cells appears to have no effect on the toxicity of DMTC at concentrations of 0.1 and 1mM.

### 3.3.2 Tolerance to Copper

The effect of copper sulphate on isolate 82Q was similar to that of DMTC (fig.3.7). Growth was totally inhibited in all phases by 1mM copper sulphate and viable cell counts indicated that this treatment rendered the cells non-viable. However, although 0.1mM copper sulphate had little effect on growth, it did lead to a decrease in the maximum population, when added at any stage in the growth cycle. The physiological age of cells of isolate 82Q thus appears to have no influence on the toxicity of copper at concentrations of 0.1 and 1mM. Cobet *et al*. (1970) studied the effect of increasing nickel concentrations on the growth of a marine *Arthrobacter*. As concentration increased over a range of 0.1-0.5mM, an increase in lag phase and a decrease in maximum population occurred. The effect of 0.1mM copper on isolate 82Q is consistent with the latter result.

The fact that the physiological age of cells of isolate 82Q has no influence on toxicity of copper or organotin might be attributed to the existence of a number of toxic effects. For instance, it has been indicated that copper may inhibit respiration; interact with proteins and render them inactive; inhibit cell division; cause changes in membrane permeability; or competitively inhibit the transport of other metals which are essential for metabolic

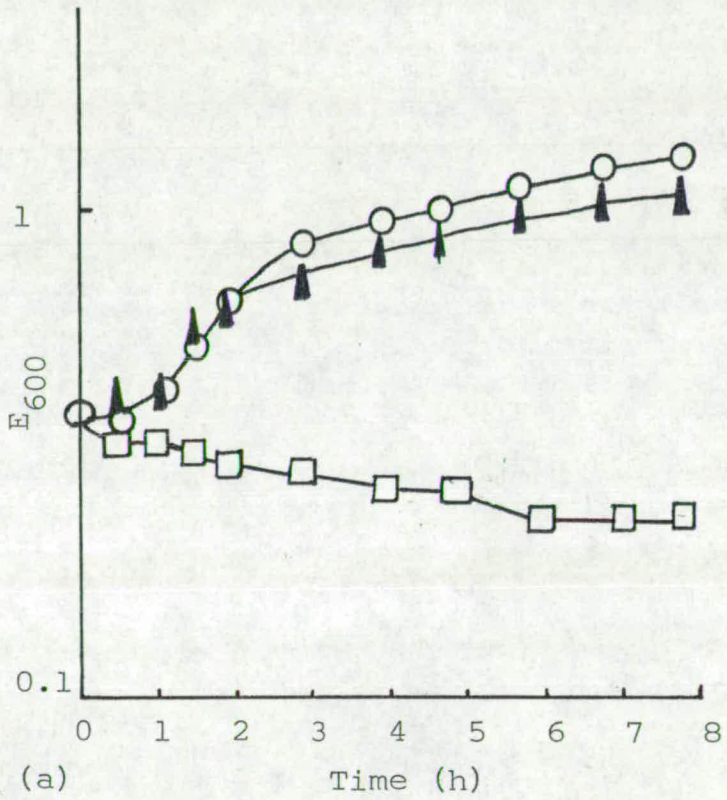
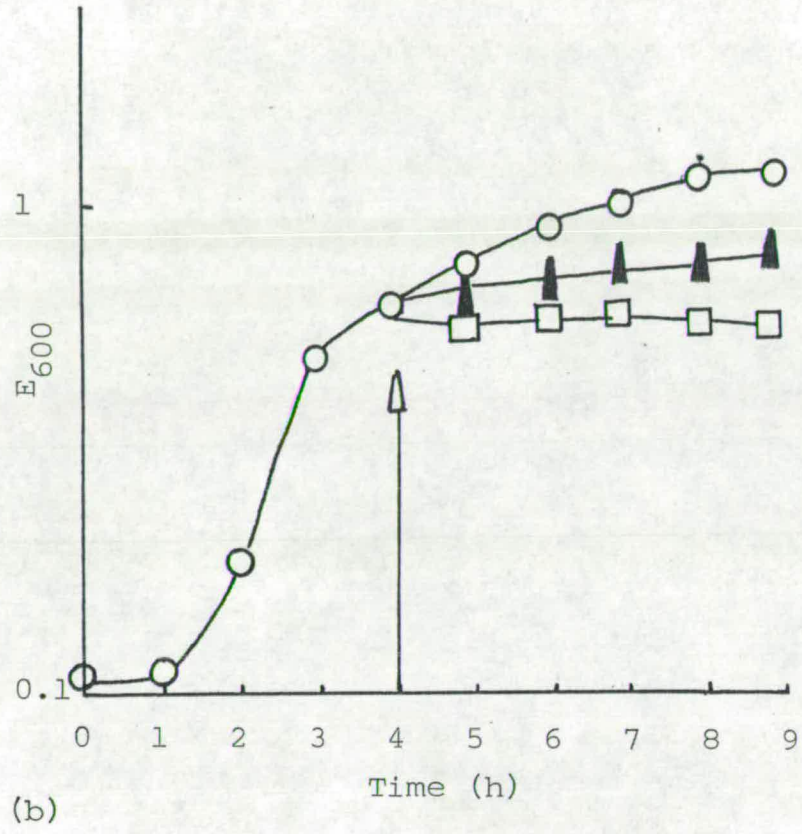
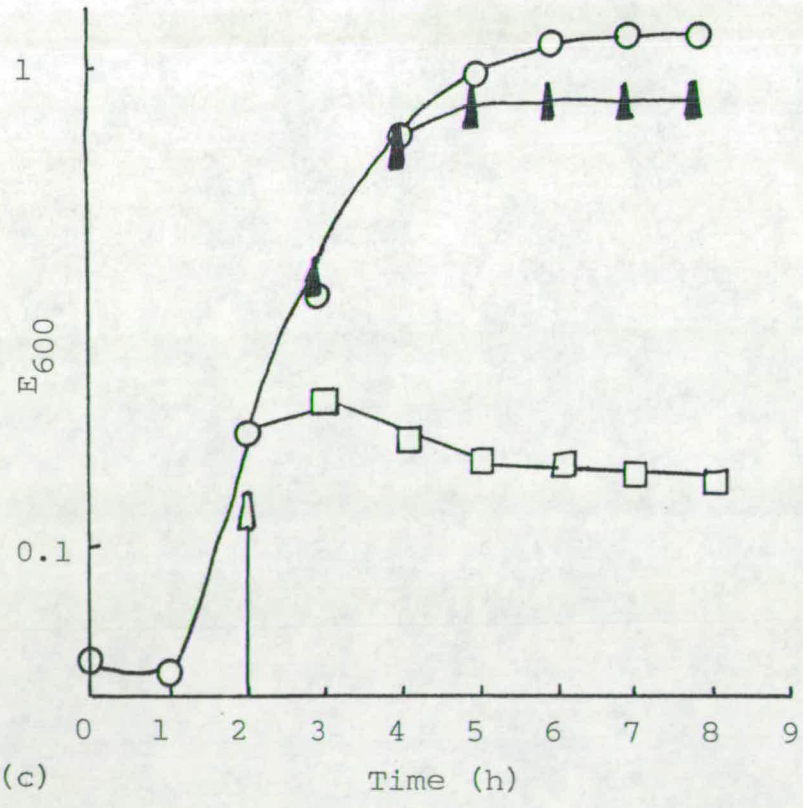



fig.3.7: Effect of copper on growth of isolate 82Q when added during lag phase (a), late logarithmic phase (b) and early logarithmic phase (c). Arrow signifies time of copper addition.

: no addition of copper
: addition of 0.1mM copper
: addition of 1.0mM copper




(b)




(c)

fig.3.7 (continued)

processes (see Section 1.8.2). Such widespread action of toxicants may ensure their toxicity at all stages of growth.

However, the limitations of these studies are obvious. Marine bacteria differ greatly in their tolerance to toxicants, thus examination of a single species is inconclusive. In addition, the toxicants were tested at only two concentrations, the greater of which totally inhibited growth. Determination of the effect of a range of concentrations between 0.1 and 1mM may have revealed further information.

Nevertheless, isolate 82Q is fairly typical of a range of marine bacteria isolated (see Section 3.1), and shows moderate resistance to copper and organotin (see Section 3.2). Thus these results may indicate that, in typical marine bacteria, there is no production of cell components exclusive to any particular growth phase which are responsible for tolerance to copper or organotin.

## 3.4 Tolerance of Cell Suspensions of Marine Bacteria to Toxicants

Morphological changes occurring in some marine isolates (see Section 3.2.3) seem to suggest that copper has an effect on the processes of cell wall synthesis and cell division. Thus, it would be expected that non-growing cells of such isolates would not exhibit morphological changes, and may show differences in their ability to tolerate toxicants.

Results have indicated that, in one marine isolate, resistance to copper and organotin is independent of the physiological age of the cells (see Section 3.3). However, these studies were carried out in growing cultures where changes in cell components would be occurring continuously. Further studies using non-growing suspensions prepared from cells at different growth phases may confirm these results.

The action of a toxicant on microorganisms must be preceded by its uptake by the cells. There appear to be two main types of metal uptake by organisms (Gadd & Griffiths, 1978). The first involves non-specific binding of the metal to the cell surface layers, whereas the second involves metabolism-dependent intracellular uptake. In most of the organisms studied, the amount of metal bound by surfaces is insignificant when compared to the amounts that can be taken up by energy-requiring processes. In the case of metabolism-dependent transport, the effect would be expected to show a greater dependence on time than in the case of non-specific metal binding.

### 3.4.1 Tolerance to Organotin

A logarithmic phase cell suspension of isolate 82Q was prepared and divided into aliquots to which were added different volumes of DMTC to give final concentrations over the range of 0.1-0.5mM. A control containing no added DMTC was included. Viable counts were performed after 3h and 24h incubations under routine conditions, and cells were observed by phase contrast microscopy. Results are shown in fig.3.8.

The effect of DMTC appears to be similar to that on growing cells of isolate 82Q (see Section 3.2.2), with little reduction in cell numbers up to 0.2mM, after which survival of cells decreased rapidly. In addition, no morphological changes were observed.

At the higher concentrations of DMTC a time-dependent effect is seen, with a greater reduction in cell survival after 24h than after 3h. This may be due to the time involved in uptake of the toxicant by the cells. In this case, cells are not metabolizing, thus uptake must be by non-specific binding of the toxicant.

### 3.4.2 Tolerance to Copper

Cell suspensions of isolates 82Q and S3/A51 were prepared. Logarithmic and stationary phase suspensions of isolate S3/A51 were divided into aliquots to which were added different volumes of copper sulphate to give final concentrations over the range of 0.2-1.0mM. A logarithmic phase suspension of isolate 82Q was treated similarly with a range of 0.1-0.5mM copper sulphate.

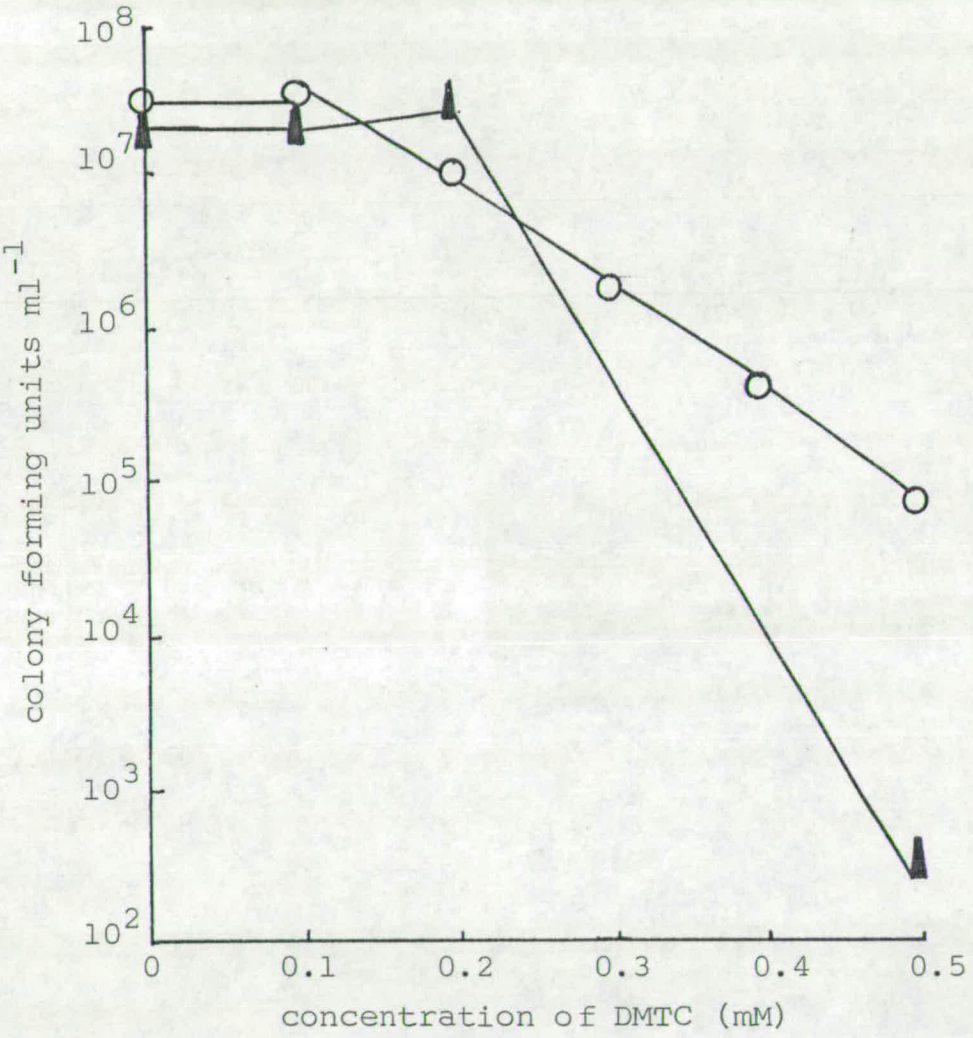
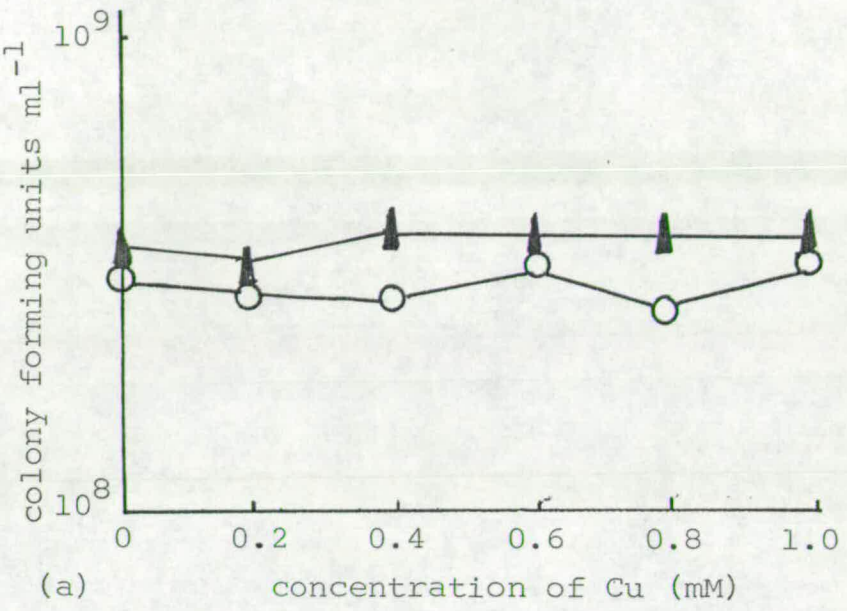



fig 3.8: Survival of logarithmic phase cells of isolate 82Q over a range of concentrations of organotin, after 3h (O) and 24h (▲) incubation periods.

Controls containing no added copper sulphate were included. Viable counts were performed after 3h and 24h incubation under routine conditions, and cells were observed by phase contrast microscopy.

Little effect was observed on survival of either logarithmic or stationary phase cells of isolate S3/A5l (fig.3.9). No morphological changes occurred and, in comparison to growing cells (see Section 3.2.2), non-growing cells appear to show greater tolerance to copper. These two results would be expected if the most significant action of copper is on the processes of cell wall synthesis and cell division. Since the outcome is similar with both logarithmic and stationary phase cells, tolerance cannot be associated with any particular components produced during an individual growth phase. Thus, as found in Section 3.3 with isolate 82Q, tolerance appears to be independent of the physiological age of the cells.

Copper ions appear to bring about a marked reduction in survival of logarithmic phase cells of isolate 82Q after 3h and 24h incubations (fig.3.10a). No morphlogical changes were observed. This effect is much greater than that on growing cultures of isolate 82Q (see Section 3.2.2), suggesting that metabolizing cells are more resistant to copper than non-growing cells. A similar experiment was performed with addition of 10mM glutamic acid to the cell suspensions to allow continuation of metabolism (fig.3.10b). This resulted in a notable difference in cell survival between 3h and 24h incubation periods. After 3h, little change in the level of cell survival occurs with increasing concentration of copper, whereas after 24h there is a gradual decrease in cell survival as copper



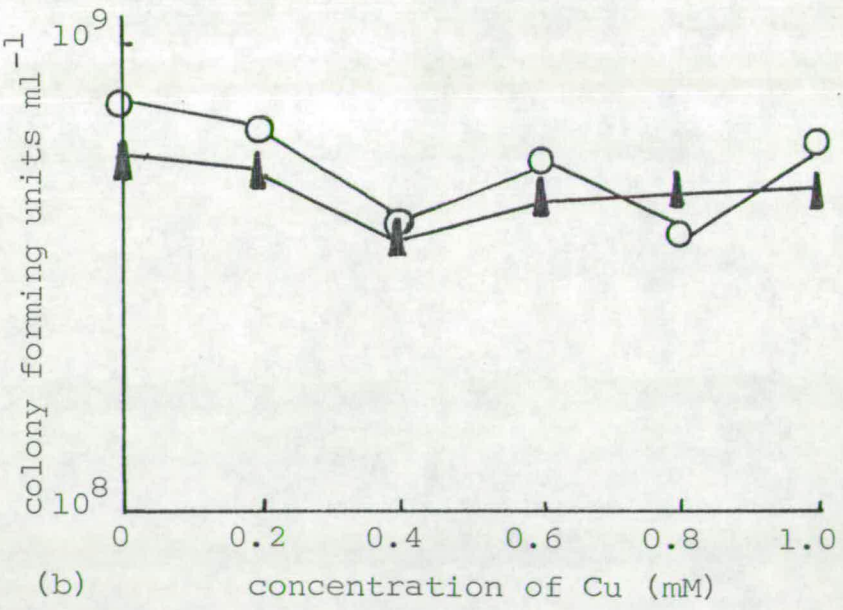
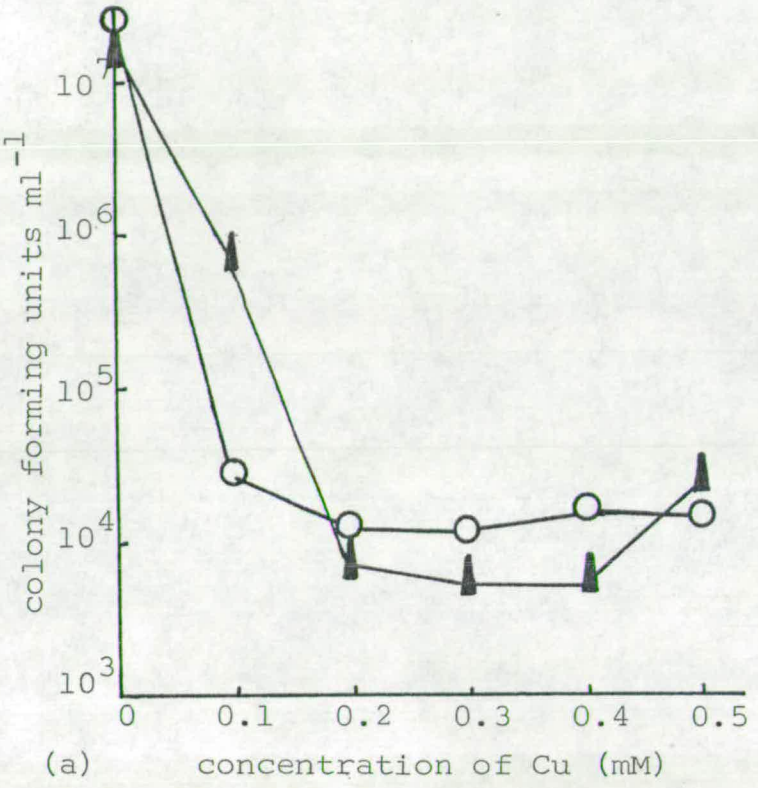




fig. 3.9: Survival of logarithmic (a) and stationary (b) phase cells of isolate S3/A51 over a range of concentrations of copper after 3h (O) and 24h (▲) incubation periods.



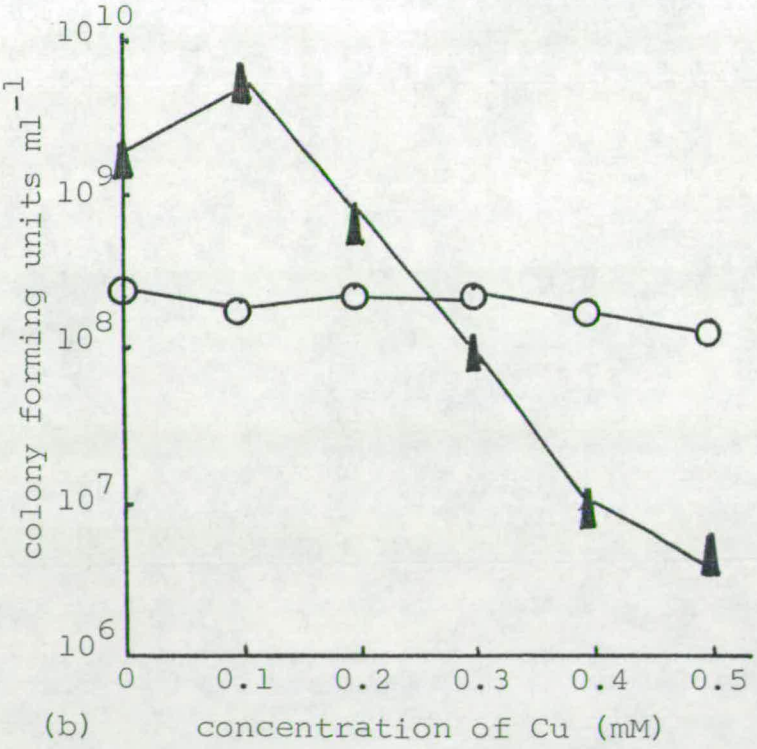




fig.3.10: Survival of logarithmic phase cells of isolate 82Q, in the absence (a) and presence (b) of 10mM glutamic acid, over a range of copper concentrations.

○ : 3h incubation period
▲ : 24h incubation period

concentration increases. Phase contrast microscopy revealed that, after 24h, both rod-shaped and spherical cells were present at the highest concentrations of copper.

The time lapse occurring before the effect of copper is seen is probably not due to the time required for uptake by the cells. This will involve metabolism-dependent intracellular uptake rather than solely non-specific metal binding to cell surface components. The latter mechanism probably accounts for the more rapid effect of copper on non-growing cells of isolate 82Q.

These results confirm that metabolizing cells of isolate 82Q are more resistant to the effects of copper than non-growing cells. This may suggest that tolerance of this isolate is inducible and is due to the production of cell components that confer resistant properties on the cell. In contrast, tolerance of isolate S3/A51, which is greater than that of isolate 82Q, does not appear to be inducible since non-growing cells are more resistant than growing cells. The greater tolerance may suggest a difference in cell permeability to copper between the two isolates. In addition this may be a reflection of differences in the mechanisms of action of copper on the two isolates. The processes of cell wall synthesis and cell division may be the most significant target for copper in isolate S3/A51, whereas another mechanism of action may be responsible for toxicity in isolate 82Q.

### 3.4.3 Tolerance to Antifouling Paint Samples

The effect of antifouling paints, containing either or both of the toxicants (see Table 2.2) previously tested, was studied by addition of glass coverslips coated with 100µl of the paint sample to logarithmic phase cell suspensions of isolates 82Q and S3/A51. Controls included suspensions to which no additions were made, and suspensions to which were added unpainted glass coverslips. Suspensions were incubated under routine conditions and viable counts were performed at various time intervals. Results are shown in figs.3.11 and 3.12.

Paint sample S3 has the greatest effect on both isolates, S2 has an intermediate effect and S1 has little effect. Reduction in cell survival is more rapid in isolate 82Q than in isolate S3/A51, the reaction occurring in hours rather than days. However, after 6d incubation there are greater numbers of surviving cells of isolate 82Q than of isolate S3/A51. Since there is little change in the cell numbers in the controls, this can be attributed to the effect of the toxicants.

Differences in the response of populations of both isolates are apparent. As incubation time increases the concentration of the toxicants in the suspension will increase due to leaching from the paint. Suspensions of isolate S3/A51 appear to contain cells which are tolerant of certain levels of toxicants, but as concentration increases with time, survival decreases rapidly. Suspensions of isolate 82Q, although showing a decrease in cell survival earlier on the time scale, and presumably at lower concentrations of toxicants,

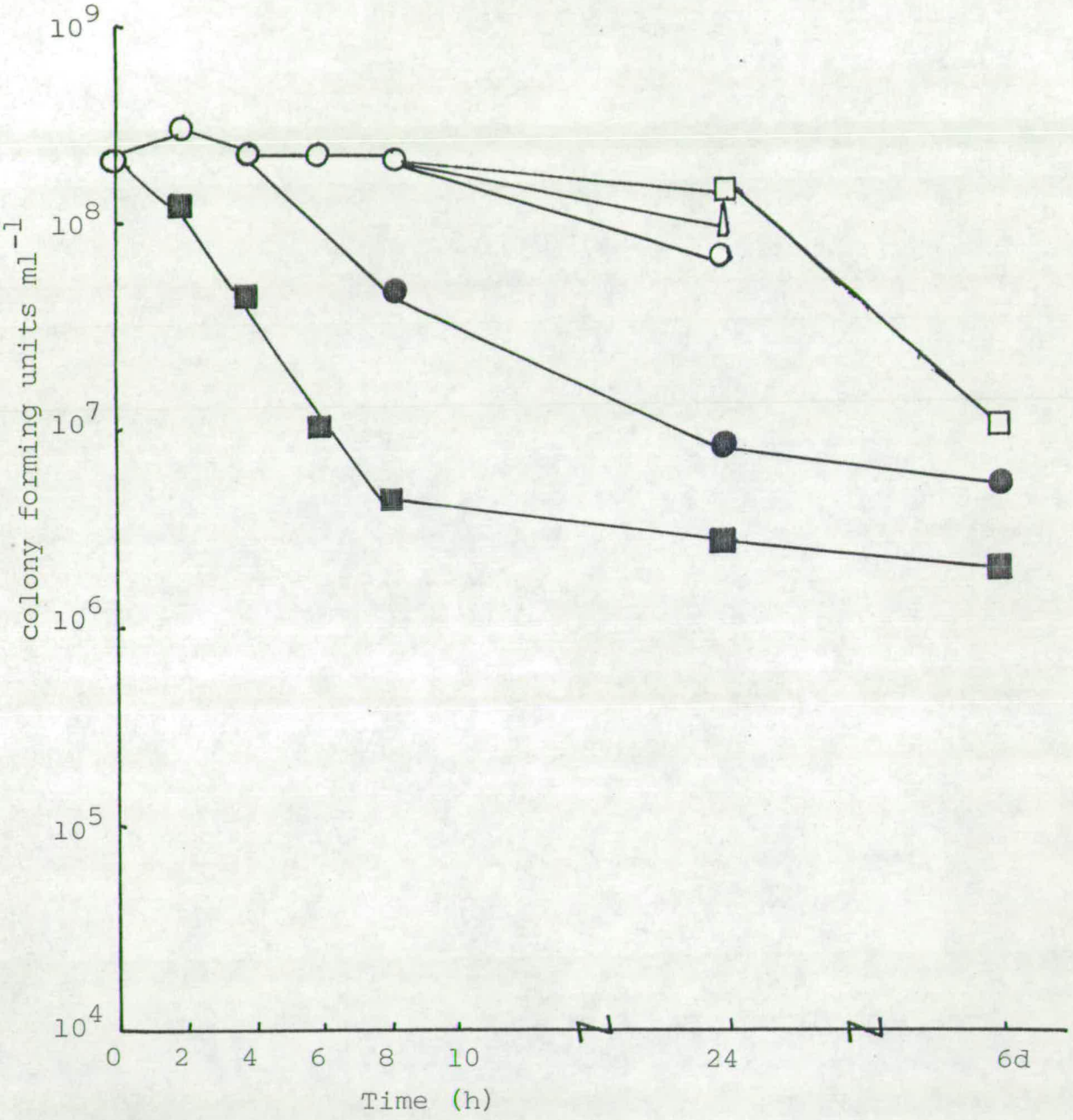



fig.3.11: Survival of logarithmic phase cells of isolate 82Q in the presence of antifouling paints.

○ : control
| : glass coverslip
□ : paint sample S1
● : paint sample S2
■ : paint sample S3

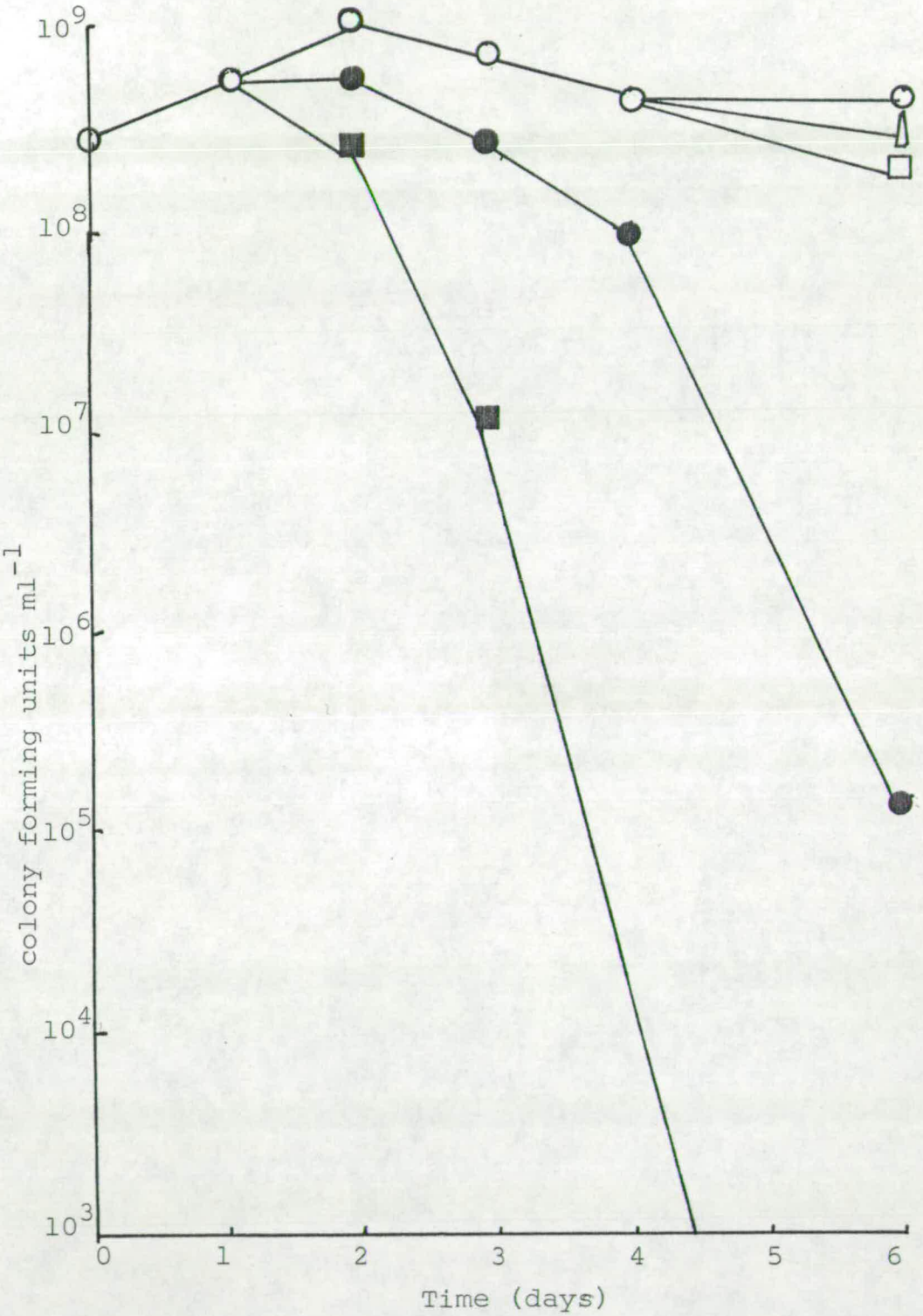



fig 3.12: Survival of logarithmic phase cells of isolate S3/A51 in the presence of antifouling paints.

○ : control
⏀ : glass coverslip
□ : paint sample S1
● : paint sample S2
■ : paint sample S3

also contain cells which are resistant even at higher concentrations of toxicants. An explanation for the greater survival of isolate 82Q may lie in the existence of naturally occurring mutants within the cell suspension, which are tolerant to one or both of the toxicants. Paint sample S2 contains organotin as the toxicant, whereas paint sample S3 contains both organotin and copper. These studies have already indicated that copper tolerance in isolate 82Q may be inducible. Thus, as cells in this experiment are not metabolizing, it is possible that the effect seen with isolate 82Q may reflect the presence of organotin-resistant mutants.

It could be argued that the response of isolate 82Q on the later part of the time scale could be due to complete leaching of the toxicants from the paint by this time, and thus a maximum concentration in the suspension being attained. However, since the same quantity of paint samples was applied in each system, and a decrease in the survival of isolate S3/A51 continues, this is unlikely.

The toxicants present in each paint sample may be responsible for their variation in effectiveness. Sample S1 contains copper, whereas sample S2 contains organotin. Thus it is perhaps surprising that sample S2 has the greater effect on both isolates, since organotin is generally found to be less effective than copper against Gram-negative bacteria (Dempsey, 1981a,b). However, this could be a reflection of the leaching rate of the toxicants from the paints, rather than of their action on the cells. It is possible that the leaching rate of copper from S1 is slower than that of organotin from S2, and that over a longer period of time S1 might

cause a greater reduction in cell survival.

Sample S3 contains both copper and organotin, thus its greater effect on both isolates may be attributed to some synergistic action of these toxicants. Babich & Stotzky (1980) indicate that interactions between toxicants may influence their toxicity. It has been proposed that organotin is ineffective against Gram-negative bacteria due to their outer membrane acting as a penetration barrier to such hydrophobic compounds (Dempsey, 1981a,b). Exposure of bacterial cells to copper may result in an increase in their permeability (Schreiber et al., 1985). Thus, when both toxicants are present, as in paint sample S3, an increased effect may result as compared to the effect of each toxicant individually. The presence of copper will result in a number of toxic effects on the cell. In addition, it may allow the penetration and thus the toxic action of organotin by causing an increase in cell permeability.

## 3.5 Attachment Studies

In recent years, many studies have been performed on the attachment of marine bacteria to surfaces (see Section 1.5), and have allowed an insight into many aspects of the subject. Some understanding of the mechanisms involved in the attachment process has been acquired (Dahlback et al., 1981; Marshall et al., 1971a), and the sequence of attachment of different bacterial types has been determined (Dempsey, 1981a,b; Marshall et al., 1971b). The association of polysaccharide with microbial attachment in aqueous environments has been demonstrated (Allison & Sutherland, 1984; Corpe, 1970b), and preliminary studies have been carried out on the nature of such polymers (Corpe, 1970b; Uhlinger & White, 1983; Sutherland, 1980). A variety of parameters including time, temperature, culture age and concentration, pH, ionic composition and shear stress have been shown to influence microbial attachment (Duddridge et al., 1981).

The acquisition of this information has relied upon the development of a range of techniques suitable for attachment studies (see Section 1.6). These vary from simple methods involving immersion of surfaces and viewing by light microscopy to the development of specialized experimental systems (Dahlback & Pedersen, 1982) and sophisticated genetic studies (Simon et al., 1985).

However, little information is available on the effects of heavy metals and organometallic compounds, such as those used in antifouling paints, on microbial attachment. Dempsey (1981a,b) has

determined the types and numbers of microorganisms colonizing copper-based and organotin-based antifouling paints in the natural marine environment over periods of up to four weeks. Copper-based paints were found to select for a range of bacteria that produce excessive amounts of extracellular polysaccharide.

Microbial attachment to a range of metal surfaces has also been studied (Berk et al., 1981; Dexter et al., 1975; Fletcher & Loeb, 1979; Marszalek et al., 1979). Dexter et al. (1975), in examination of bacterial attachment to a number of surface types, found that the rate of attachment was slowest for copper surfaces . Copper-nickel alloys were reported to be selective for bacteria which excrete extracellular mucoid material (Marszalek et al., 1979). In addition, these surfaces fouled at a slower rate and were characterized by a less diverse microfouling community.

A number of reports indicate varying influences of cations on microbial attachment to surfaces (see Section 1.3.3). In particular Duddridge et al (1981) demonstrated that bacterial adhesion was significantly reduced by a range of metal cations, including copper ions.

### 3.5.1 Effect of Incubation Time

Attachment assays were carried out as described in Section 2.8, with enumeration of attached and suspended cell populations at timed intervals over a 24h period. Results obtained using isolate 82Q are shown in fig.3.13. Similar trends were found with both isolates 82B and S3/A51 (not shown). Initially, attachment occurred very rapidly,

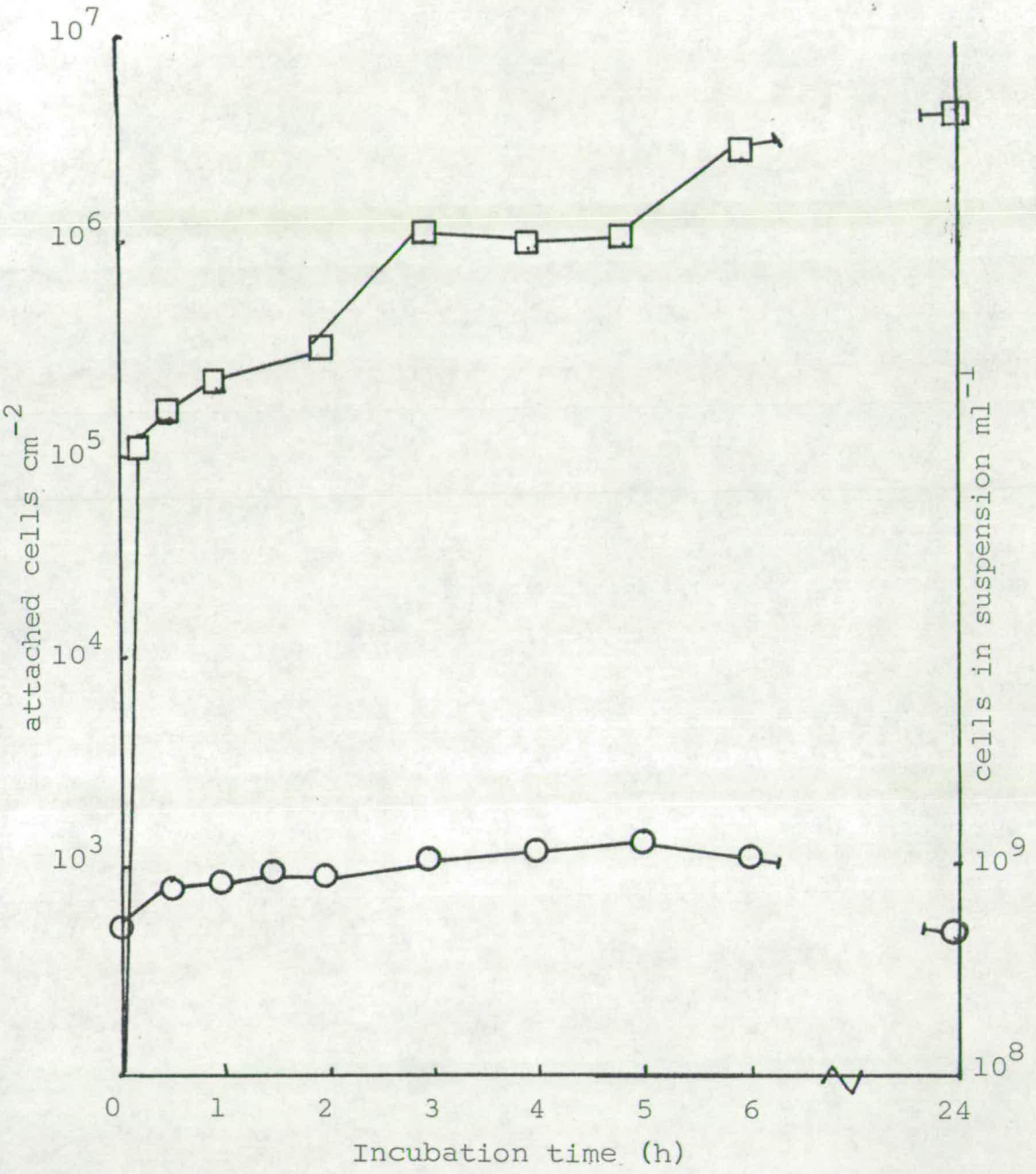



fig.3.13: Attachment of isolate 82Q to glass slides over a 24h period.

○ : number of cells in bacterial suspension
□ : number of cells attached to the glass surface.

with $10^5$ cells $cm^{-2}$ becoming attached to the glass surface within an incubation period of 10 min. This was followed by a more gradual increase in numbers of attached cells over the 24h period. During this time the numbers of cells in suspension remained fairly constant.

Visualization of attached bacteria by staining and light microscopy revealed the progressive build up of cells on the glass surface (Plate 4). The staining technique used (see Section 2.10.1) allowed visualization of attached cells and their associated exopolysaccharides. Attached cells were observed to increase in numbers with time, eventually resulting in the formation of microcolonies surrounded by exopolysaccharide.

The dependence of attachment upon time would be expected, as an increase in time leads to an increase in the number of encounters of cells with the surface and thus an increase in the chance of attachment occurring. Similar results have been obtained in studies using non-growing cell suspensions of pseudomonads (Duddridge et al., 1981; Fletcher, 1977; Stanley, 1983). It is presumed that, as surfaces were rinsed vigorously to remove weakly attached cells, the irreversible phase of attachment is being studied. The rapid attachment observed thus indicates that irreversible attachment may occur very quickly. Since cells in this study are not metabolizing, they must then exist in a condition already prepared for the attachment process. This agrees with the work of Stanley (1983), who reports that attachment of cells began with an incubation time of less than 1 min., making it unlikely that any structural or metabolic changes in the cell are necessary. However, further synthesis of extracellular polymer probably occurs after attachment.

Plate 4: Visualization of attachment of cells of isolate 82Q to glass slides and build up of associated extracellular polysaccharide over a 24h period.

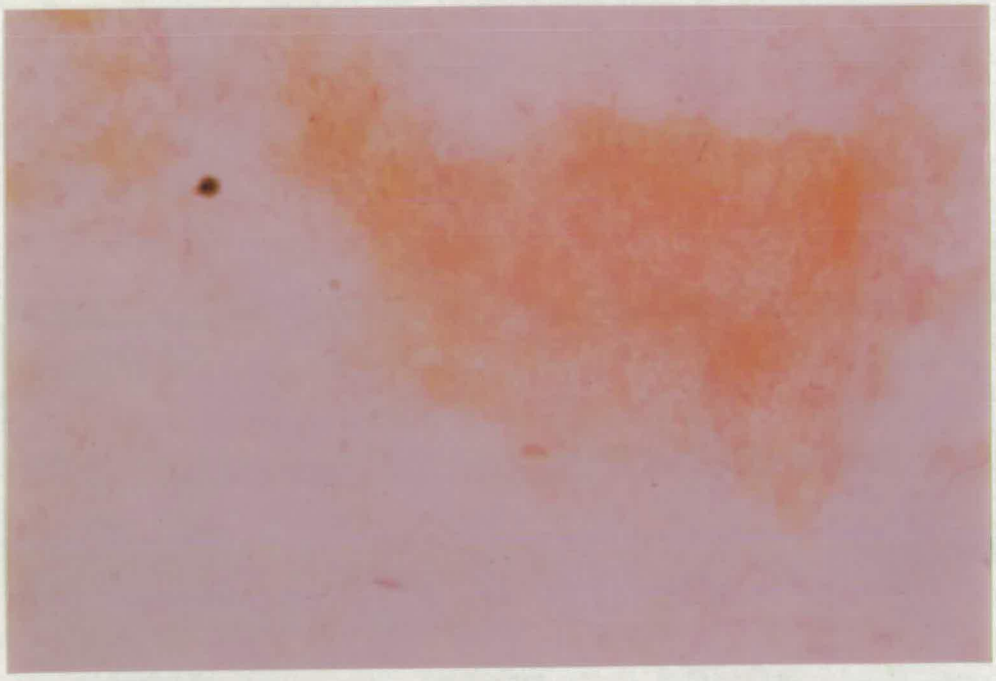
(a) 1h incubation; (b) 3h incubation;
(c) 6h incubation; (d) 24h incubation.

(a)

(b)

Plate 4

(c)



(d)

Plate 4 (cont'd)

The development of microcolonies has been reported in attachment studies using growing cultures (Ellwood et al., 1982). It is proposed that adsorption of single cells and their subsequent growth at the surface leads to microcolony formation. However, in the case of non-growing cells the development of microcolonies cannot result from surface growth. It is probable that attachment of single cells and their extracellular polymers at sites on the surface changes the properties of those areas. This could result in an increase in the tendency for bacteria to attach at these sites, and the appearance of microcolonies.

The simple technique used in these attachment assays is similar to that used in many other studies. In preparation of cell suspensions, some workers wash and respin the harvested cells before resuspending in buffer (Stanley, 1983). However, it has been reported that the number of cell washings has a considerable influence on attachment ability due to removal of essential cell surface components (Bright & Fletcher, 1983). Thus this procedure was omitted in these experiments.

The method of enumeration of attached cells has its disadvantages. Swabbing may result in cells being lost due to breakage or incomplete removal, and plate counts may be unreliable due to clumping of cells. Thus the results obtained represent only a minimum of the attached population (Costerton, 1980). However, as duplicate assays yielded similar results, and only the general trend in the rate of attachment is required rather than specific numbers, this technique is sufficient for these studies. To obtain a more comprehensive picture of bacterial attachment, data can be confirmed

using other systems, for example, direct microscopic counts or radiolabel assays (Mackowiak & Marling-Cason, 1984).

The temperature of incubation for these attachment studies was 30°C. Temperature, which has a fundamental influence on reaction rate and microbial metabolic activity, has been observed to affect attachment (Duddridge et al., 1981; Fletcher, 1977). Fletcher (1977) offers three possible explanations for the decrease in attachment with a lowering in temperature. Firstly, attachment might be reduced at low temperature due to an accompanying increase in the viscosity of the medium or of the bacterial surface polymers. Secondly, higher temperatures (within limits) favour chemisorption, and if this process determines initial bacterial attachment, decreased attachment at lower temperatures may thus be explained. Finally, temperature may influence attachment by affecting the physiology of the organisms. However, although a decrease in the numbers of attached cells occurred at lower temperatures, the same general trend was observed in the attachment process at both 3°C and 20°C.

### 3.5.2 Effect of Organotin

Attachment assays were performed on isolates 82Q and S3/A51, with DMTC added to a number of cell suspensions to give final concentrations over the range of 0.1-0.8mM. Controls containing no added organotin were included. Enumeration of attached and suspended cells was carried out after an 18h incubation under routine conditions.

DMTC caused a gradual decrease in both survival and attachment

of cells of isolate 82Q at concentrations of up to 0.5mM (fig.3.14). As concentration increased above this point, a more dramatic reduction in survival and viability of attached cells followed. Attachment appeared to be related to cell survival, which may suggest that DMTC decreases attachment of cells of 82Q by rendering them non-viable. However, the mechanism of enumeration employed accounts for viable cells only, thus cannot determine if non-viable cells retain the ability to attach.

Enumeration by direct microscopic counting has been used in studies on the attachment of dead cells to surfaces (Stanley, 1983). Cells of Pseudomonas aeruginosa killed by heating or formaldehyde were capable of reduced but significant attachment. However, Meadows (1971) found that, although bacteria killed by ultraviolet radiation attached to surfaces in only slightly reduced numbers compared to viable controls, the numbers of heat and formaldehyde killed bacteria that attached were very low. Ultraviolet radiation kills cells by inactivating DNA thus, if attachment depends on the integrity of the outer surface, it should be unaffected by UV-induced death. The reduction in attachment of heat and formaldehyde killed cells could be explained in terms of alterations to the cell surface layers by these treatments causing a decreased tendency to attach. Since metals are known to interact with cell surface components (see Section 1.8.1), it is likely that organotin may cause a reduction in attachment by a similar process, which may also be responsible for killing the cells.

A different trend was observed in the case of isolate S3/A51 (fig.3.15). The number of cells in suspension remained fairly constant over the concentration range. However, with 0.1mM DMTC, the

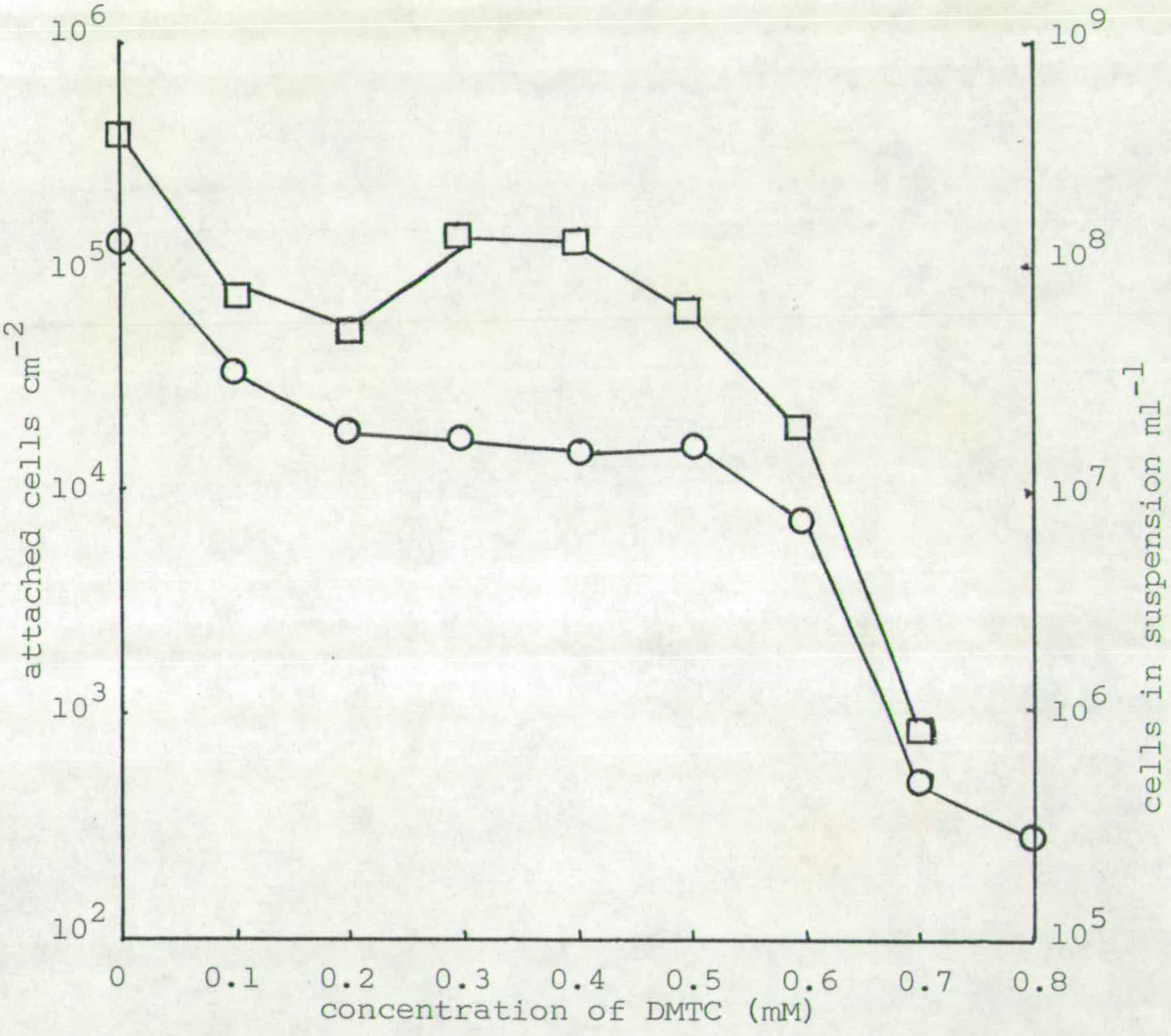



fig.3.14: Effect of a range of concentrations of organotin on survival (O) and attachment to glass slides (□) of isolate 82Q.

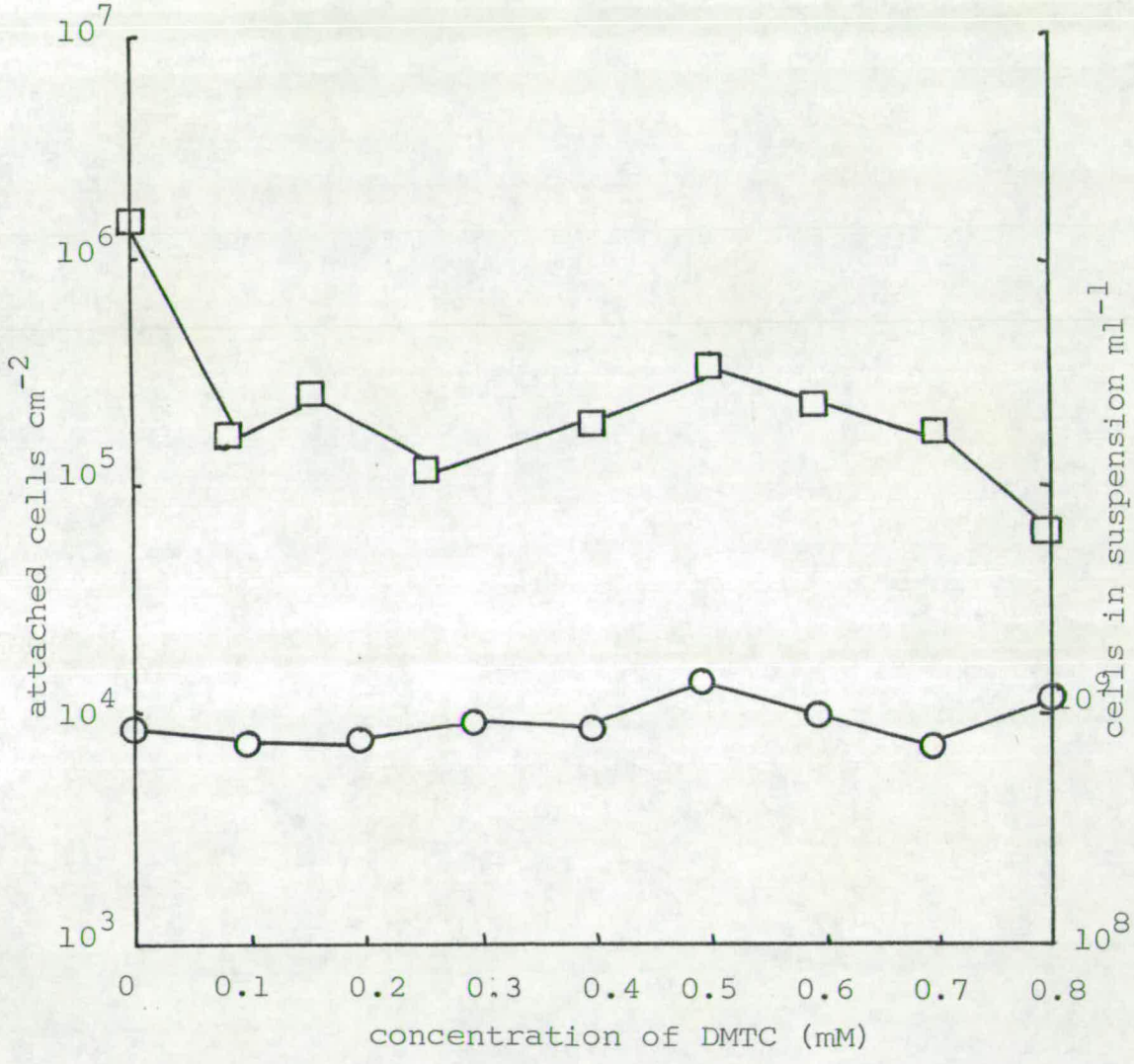



fig.3.15: Effect of a range of concentrations of organotin on survival (O) and attachment to glass slides (□) of isolate S3/A51.

number of attached cells was reduced 10-fold, though little further reduction occurred at higher concentrations. Thus, unlike isolate 82Q, viability of attached S3/A51 in the presence of DMTC does not appear to be related to cell survival. These results may indicate that DMTC interacts with cell surface components of isolate S3/A51, produced under routine growth conditions (i.e. in the absence of DMTC), resulting in a reduction in the tendency for attachment to occur, without causing a loss of viability.

### 3.5.3 Effect of Copper

Attachment assays were performed with the addition of copper sulphate to a number of cell suspensions to give final concentrations over the range of 0.1-0.8mM for isolate 82Q and 0.2-1.6mM for isolate S3/A51. Controls containing no added copper sulphate were included. Enumeration of attached and suspended cells was carried out after an 18h incubation under routine conditions.

At a concentration of 0.1mM, copper sulphate caused a marked reduction in both survival and viability of attached isolate 82Q (fig.3.16). As the concentration was increased further, the numbers of cells surviving and attaching levelled out. These results suggest that, as in the presence of DMTC, attachment of isolate 82Q in the presence of copper is related to cell survival. Copper may interact with surface components of these cells, reducing their ability to attach and rendering them non-viable.

Isolate S3/A51 showed little change in cell survival over the range of 0-1.6mM copper sulphate (fig.3.17). The presence of 0.2mM

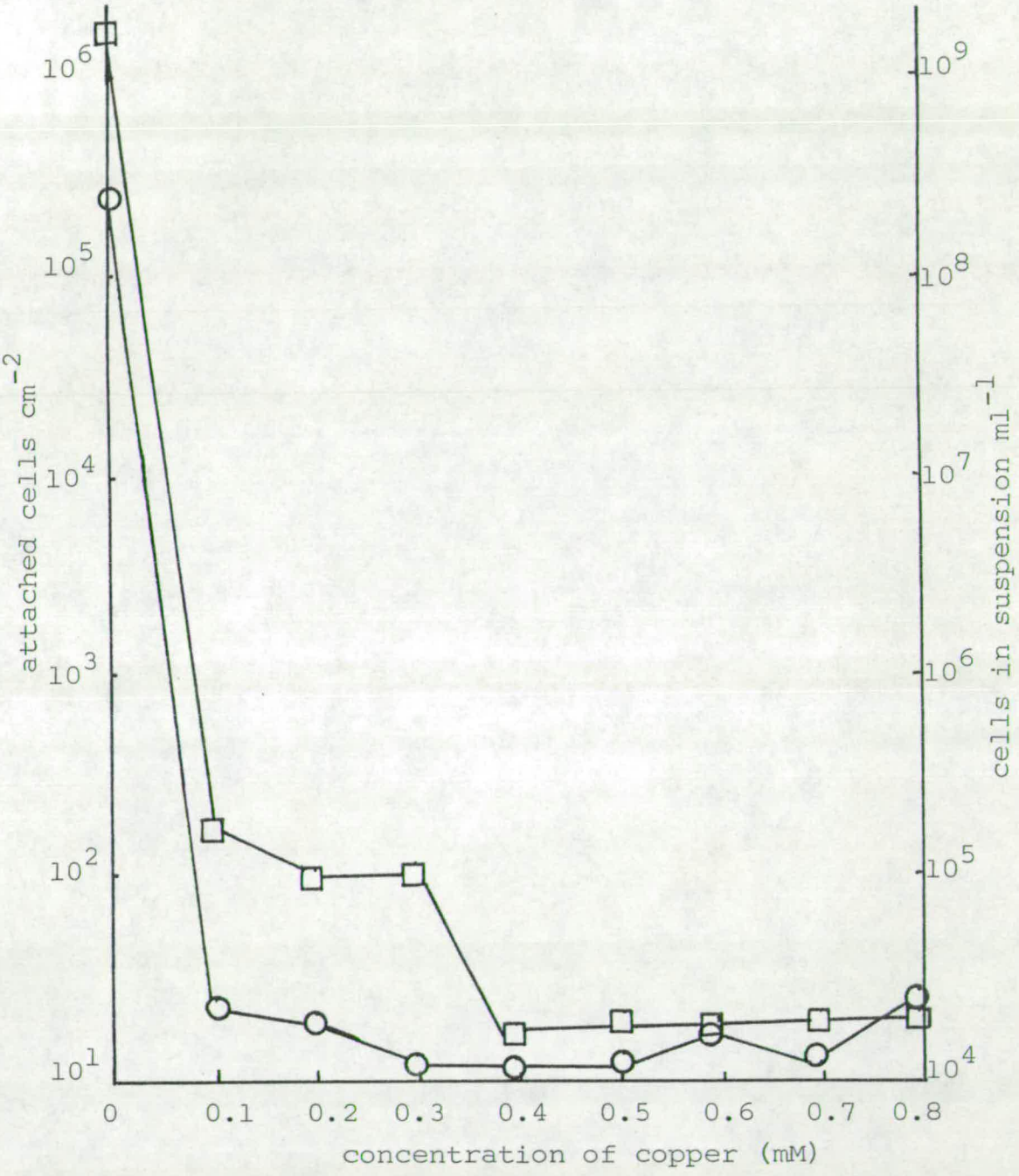



fig.3.16: Effect of a range of concentrations of copper on survival (○) and attachment to glass slides (□) of isolate 82Q.

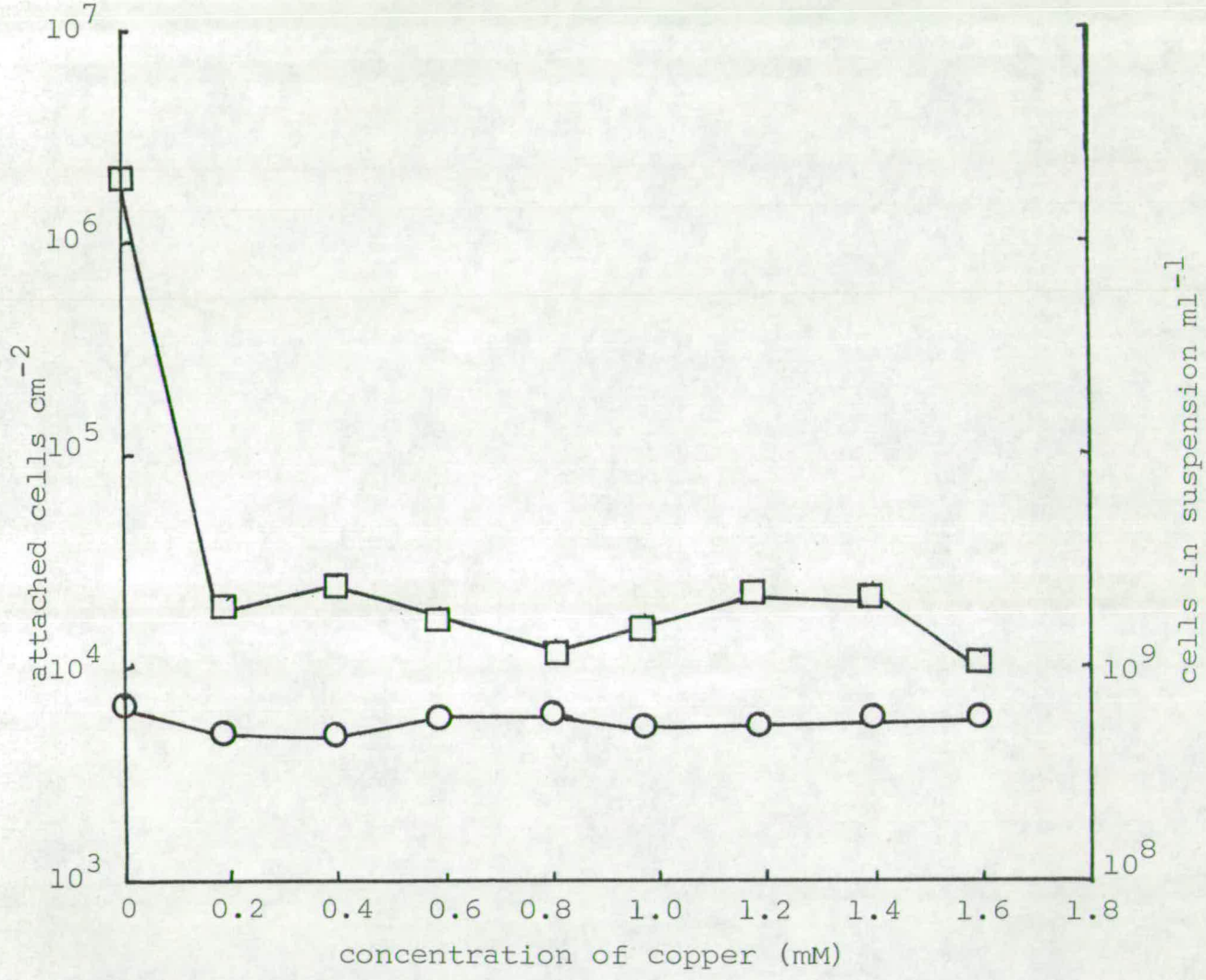



fig.3.17: Effect of a range of concentrations of copper on survival (○) and attachment to glass slides (□) of isolate S3/A51.

copper sulphate caused a 100-fold reduction in the numbers of attached cells, whereas at higher concentrations little further reduction was observed. This is similar to the effect observed with DMTC, and may be explained in similar terms. Copper ions may interact with cell surface components required for the attachment process, thus reducing attachment to surfaces. However, as logarithmic cells of isolate S3/A51 show tolerance to copper over this range of concentrations (see Section 3.4.2), no loss of viability is incurred. Binding of copper to the anionic surfaces of bacterial cells may alter the surface charge, converting it from electronegative to electropositive (Beveridge & Koval, 1981). Since bacterial surface charge may have an influence on attachment properties (see Section 1.4.1), this could offer an additional explanation for reduced attachment. Glass surfaces were used in these studies and these will also supply an anionic surface to which copper can bind. This process may result in alteration of the charge on the glass surface, which would have an influence on attachment.

Mutant 82Q/15b was tested to determine whether it exhibited increased cell survival and attachment in the presence of copper, as compared to the parental strain 82Q. Cells previously grown under routine conditions (i.e. in the absence of copper) were used in attachment assays, with the presence of copper sulphate to give final concentrations over the range 0.3-1.0mM. Results are shown in fig.3.18. Reduction in both survival and attachment of cells occurs more gradually than that of the parental strain, though the same general trend is observed. However, the numbers of attached and suspended cells observed at the higher concentrations of copper is

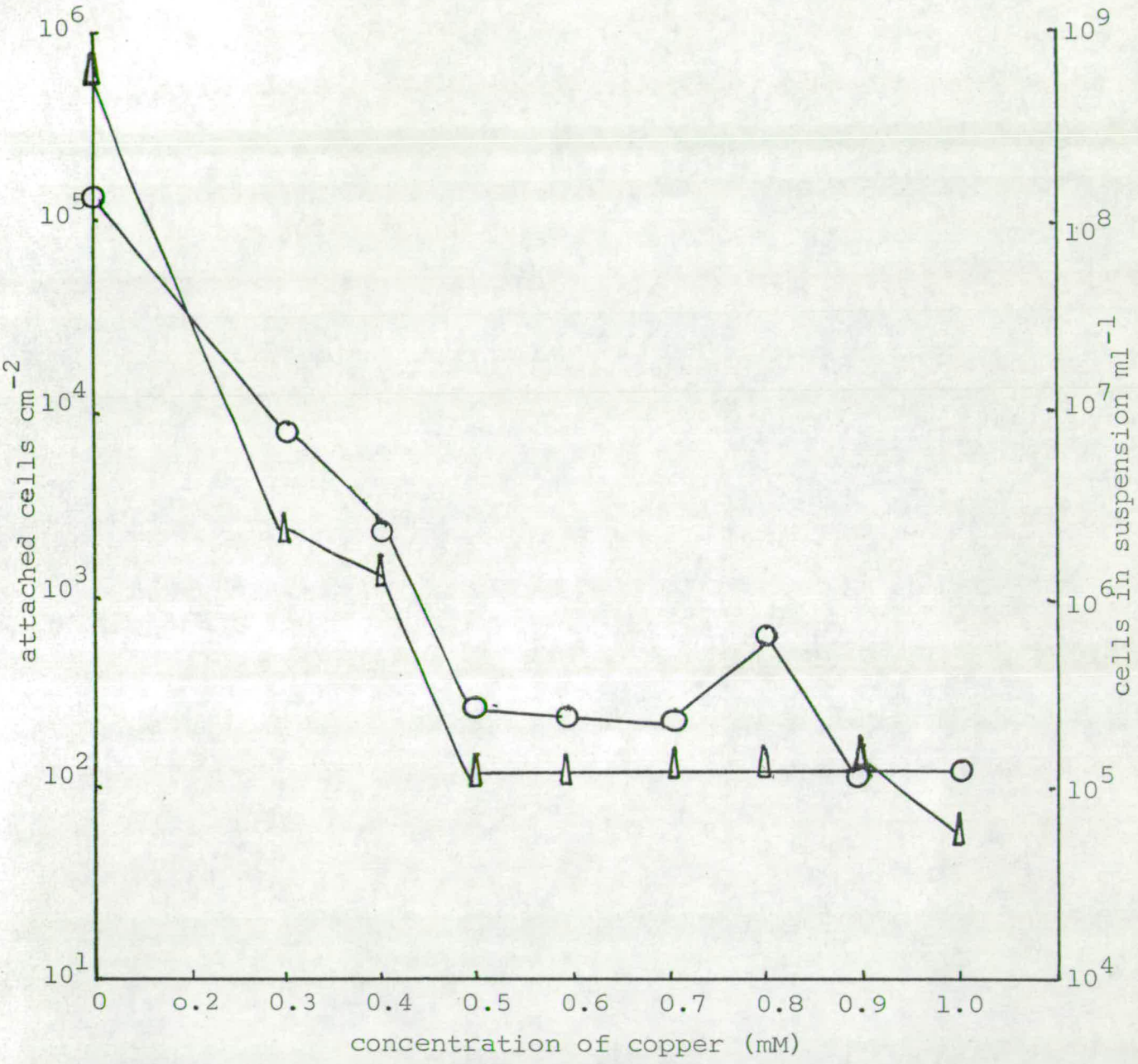



fig.3.18: Effect of a range of concentrations of copper on on survival (O) and attachment to glass slides (Δ) of mutant 82Q/15b, previously grown under routine conditions.

greater with the mutant strain than with the parental strain. Thus, as counts obtained in the control assays of each experiment are similar, these results suggest that non-growing logarithmic phase cells of mutant 82Q/15b are more tolerant to copper than those of isolate 82Q.

Cells of mutant 82Q/15b which had previously been grown in media containing 0.5mM copper sulphate were used in a similar assay (fig.3.19). A more gradual reduction in cell survival over the range of concentrations suggests that, during growth of this mutant in the presence of copper ions, some cell component is produced which confers tolerant properties on the cell. This implies an inducible tolerance mechanism. The observed reduction in attachment, particularly from the control to 0.2mM copper sulphate, does not appear to be related to cell survival. This is similar to the effect of copper observed on isolate S3/A51, and can probably be explained by the interaction of copper ions with cell surface components resulting, in some way, in a reduction in the tendency to attach to surfaces.

Extracellular polymeric material has been implicated in microbial adhesion to surfaces (see Section 1.4.7), and in the binding of metal cations (Cassity & Kolodziej, 1984; Corpe, 1975), thus making them more readily available to the cell surface for transport into the cell. Possibly, these observations could explain the effects of toxicants on cell survival and attachment. Interaction of toxicants with extracellular polymeric material could result in an alteration of its properties and thus a reduction in attachment. In addition, the greater availability of toxicants in the vicinity of the cell surface for action on the cell could result in a decrease in cell survival.

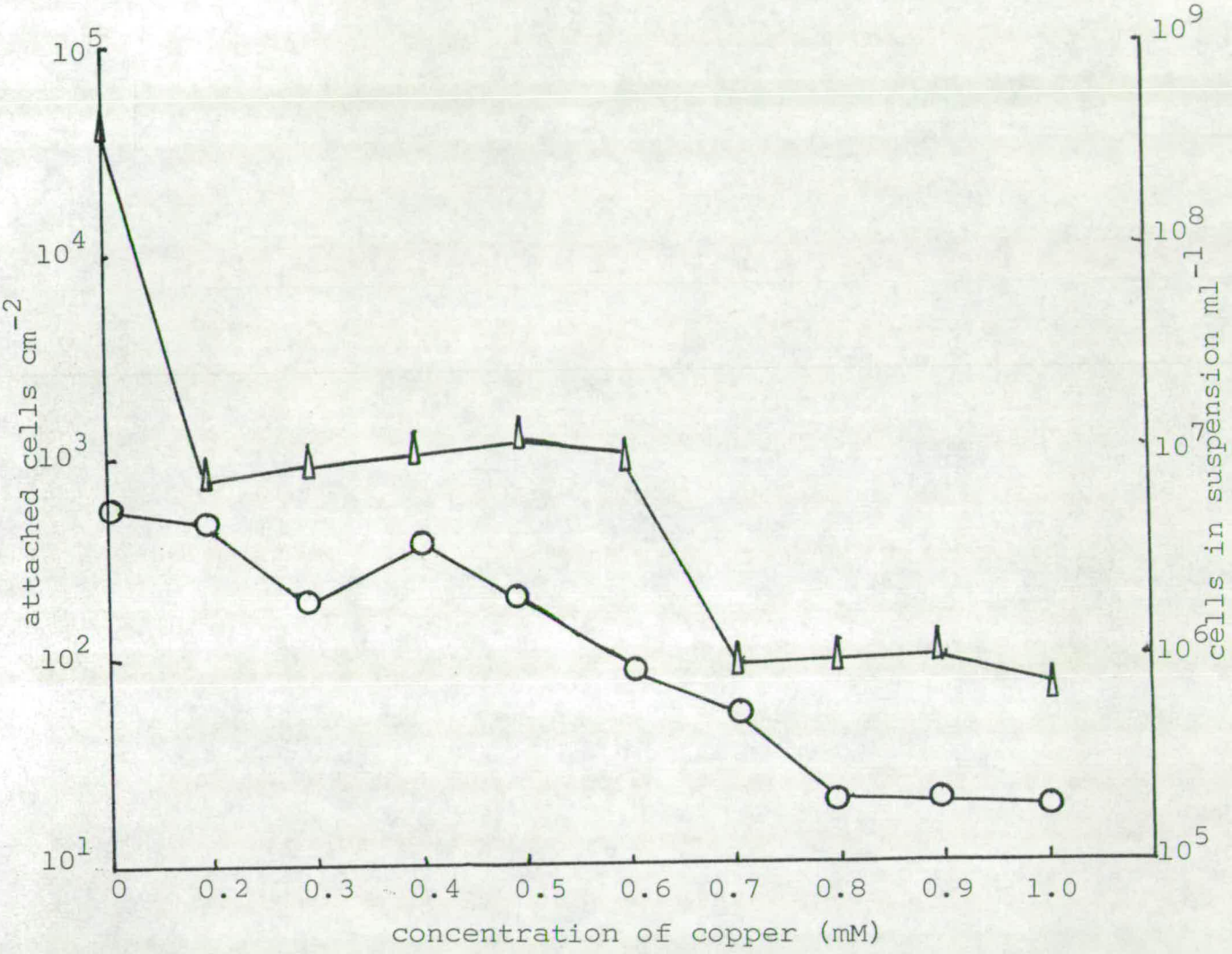



fig.3.19: Effect of a range of concentrations of copper on survival (O) and attachment to glass slides (Δ) of mutant 82Q/15b, previously grown in the presence of 0.5mM copper sulphate.

## 3.6 Effect of Toxicants on Exopolysaccharides Produced by Marine Bacteria

It has been suggested that the action of toxicants, such as copper and organotin, on marine bacteria, and the tolerance of some isolates to these toxicants is dependent on their interaction with cell surface components (see Section 3.5). Bacterial exopolysaccharides have been shown to bind metal cations, including copper (Corpe, 1975; Manzini et al., 1984), and may influence their permeation into the cell.

Dempsey (1981a,b) has demonstrated that bacteria attaching to copper-based antifouling paints produce copious amounts of polysaccharide. It is not clear whether this substrate selects for excessive polysaccharide producers or induces tolerant strains to secrete excessive amounts of polysaccharide. In either case, this indicates that the production of excessive amounts of polysaccharide provides some form of protection from the toxic effects of copper, perhaps due to physical blockage of the paint matrix. The polysaccharide would thus act as a diffusion barrier causing a reduction in the leaching rate of the toxicant from the paint. However, Corpe (1975) found no evidence that marine bacteria exhibiting tolerance to toxic metal cations produced larger quantities of extracellular polymers when grown in the presence of added metal than in its absence.

Cassity & Kolodziej (1984) suggest that extracellular polymers produced by many bacteria are capable of binding cations, thus making them more readily available to the cell surface for transport

into the cell. They propose that this would be of advantage to organisms, since they could better tolerate small amounts of toxic metals in their environment or retain necessary metallic ions near the cell surface if they were in an environment containing few metallic ions.

The chemical and physical nature of exopolysaccharides produced by fouling bacteria has not yet been elucidated. It would be of particular interest to determine any differences between those produced by toxicant-sensitive and toxicant-tolerant strains. Properties conferred by changes in the structure of the polysaccharide may be involved in tolerance to toxicants or in attachment to surfaces. For instance, interactions of exopolysaccharides with cations resulting in the formation of gels, flocs or precipitates may have an influence on their proposed role in attachment (Sutherland, 1982). The presence of acetate or pyruvate groups, or uronic acids would also have an effect on the properties of the polysaccharide. Manzini et al(1984) demonstrated that bacterial polysaccharides formed complexes with divalent copper ions which involve an intimate interaction between a negative $COO^-$ donor function and the metal ion. An increase in acetate, pyruvate or uronic acids would increase the negative charge of the polymer and thus increase its ability to chelate metal ions. A change in the charge of extracellular polysaccharide could result in a change in the overall surface charge of the bacterial cell, which might affect the tendency to attach to surfaces (see Section 1.4.1). In addition, there may be some alteration in the rheological properties of the polymer, which could influence attachment.

## 3.6.1 Chemical Composition

### 3.6.1.1 Monosaccharide Constituents

Analysis by acid hydrolysis and paper chromatography of exopolysaccharides produced by isolates 82B, 82Q and S3/A51 revealed the nature of the monosaccharide constituents (see Table 3.5). No differences in these components were detected in polysaccharides produced during growth in the presence of 0.7mM copper sulphate or 0.1mM DMTC (82B); 0.1mM copper sulphate or 0.1mM DMTC (82Q); and 1.0mM copper sulphate or 0.2mM DMTC (S3/A51). In addition, mutant 82Q/15b produced a polymer with the same monosaccharide constituents as the parental strain 82Q, and these components remained unchanged during growth in the presence of 0.8mM copper sulphate.

Analysis by HPLC yielded more information on the polysaccharides of isolates 82Q and S3/A51. This technique has the advantages that it allows rapid analysis and optimized separation of the residual components. In addition, it is able to detect trace components, which are not detected by paper chromatography.

Exopolysaccharide produced by isolate 82Q was again shown to contain the same monosaccharide constituents during growth in the presence or absence of toxicants. However, small differences were observed in the molar ratios of these components. In polysaccharide produced in the absence of toxicants, and in the presence of 0.1mM DMTC, the molar ratio of glucose: galactose: rhamnose: fucose was calculated to be 1.0: 1.1: 0.2: 0.1. In the presence of 0.1mM copper, the molar ratio was calculated as 1.0: 0.9: 0.2: 0.2. In the

case of isolate S3/A5l, in addition to the monosaccharide components detected by paper chromatography, trace amounts of rhamnose were detected by HPLC analysis. Again, no changes in these constituents were seen during growth in the presence or absence of toxicants, but differences in the molar ratios were more apparent. In the absence of toxicants the molar ratio of glucose: galactose: rhamnose: fucose was 1.0: 1.6: 0.1: 1.3. However, in the presence of 1.0mM copper the molar ratio was found to be 1.0: 2.2: 0.1: 1.3, showing an increase in the ratio of galactose. In the presence of 0.2mM DMTC, the molar ratio was calculated as 1.0: 0.9: 0.3: 0.8, showing variations in all components as compared to glucose. The molar ratios obtained in these studies suggest that if the polysaccharides examined contain repeat structures, these units must be large. There is a possibility that the trace amounts of rhamnose and fucose in extracellular polysaccharide from isolate 82Q, and rhamnose in that from isolate S3/A5l, might be contaminants from LPS.

These results would imply that the presence of divalent copper ions exerts some influence on the biosynthetic pathway of polysaccharides in the case of both isolates. Differences in the ratio of monosaccharide constituents may result in differences in the structure of the polysaccharide. It has been suggested that polysaccharide structure rather than chemical composition is responsible for adhesiveness in aquatic bacteria (Sutherland, 1980; 1982). Thus, particularly in the case of isolate S3/A5l, the difference in the ratio of monosaccharide constituents in the presence of copper may result in a change in the structure of the polysaccharide such that its adhesive properties decrease. In

addition, a change in molecular mass of the polysaccharide could lead to different viscosity characteristics which might also affect adhesive properties. Thus, growth in the presence of copper may result in a decrease in attachment without a related decrease in viability of cells of isolate S3/A5l.

3.6.1.2 Carbohydrate Moiety

The total carbohydrate and protein content of polymers produced by isolates 82B, 82Q and S3/A5l is shown in Table 3.7. Low values of protein were expected due to adsorption by ion-exchange resin in the purification procedure. The highest value obtained for carbohydrate content was 74% (w/w) polymer. The variation in these values may be due to incomplete lyophilization of the polymer.

Variations in the quantities of acyl components of polymers produced during growth in the presence and absence of toxicants were observed (table 3.8). Isolate S3/A5l showed some increase in amounts of acetate and uronic acid in the presence of toxicants. This may increase the tendency of the polymer to bind the toxicants, and might suggest that chelation of toxicants by extracellular polysaccharides is involved in tolerance mechanisms.

The major differences in polymers produced by isolate 82Q were in the uronic acid content, which was greatly reduced in the presence of toxicants. Some increase in the quantities of acetate occurred concomitantly. The mutant 82Q/15b also had reduced amounts of uronic acid compared to the parental strain. However, this quantity remained unchanged in the presence of copper, and

Table 3.7: Chemical Composition of Extracellular Polysaccharides from Marine Isolates

| Isolate | Growth Medium | Carbohydrate | Protein |
|---|---|---|---|
| 82Q | MIIYE | 74 | 0.9 |
| | MIIYE+0.1mM Cu | 64 | 1.5 |
| | MIIYE+0.1mM DMTC | 70 | 2.2 |
| 82Q/15b | MIIYE | 51 | 2.1 |
| | MIIYE+0.8mM Cu | 35 | 1.6 |
| 82B | MIIYE | 45 | 1.4 |
| | MIIYE+0.7mM Cu | 47 | 1.7 |
| | MIIYE+0.1mM DMTC | 50 | 0.6 |
| S3/A51 | MIIYE | 65 | 0.5 |
| | MIIYE+1mM Cu | 63 | 0.7 |
| | MIIYE+0.2mM DMTC | 64 | 0.4 |

Values are expressed as % (w/w) of lyophilized polymer

Table 3.8: Composition of the Carbohydrate Moiety of Extracellular Polysaccharides from Marine Isolates

| Isolate | Growth Medium | Acetate | Pyruvate | Uronic Acid |
|---|---|---|---|---|
| 82Q | MIIYE | 3.4 | 0.5 | 15.3 |
| | MIIYE+0.1mM Cu | 9.8 | 0.2 | 2.7 |
| | MIIYE+0.1mM DMTC | 6.1 | 0.3 | 3.4 |
| 82Q/15b | MIIYE | 2.4 | 1.4 | 4.7 |
| | MIIYE+0.8mM Cu | 3.1 | nd | 4.8 |
| 82B | MIIYE | 5.1 | nd | nd |
| | MIIYE+0.7mM Cu | 3.8 | nd | nd |
| | MIIYE+0.1mM DMTC | 1.2 | nd | nd |
| S3/A51 | MIIYE | 10.2 | 4.0 | 10.6 |
| | MIIYE+1mM Cu | 13.5 | 4.3 | 14.4 |
| | MIIYE+0.2mM DMTC | 13.0 | 4.7 | 17.2 |

Values are expressed as % (w/w) of carbohydrate content

nd: not done

there was little apparent change in the quantities of acetate.

The reduction in uronic acid content in the presence of toxicants would result in a polymer with a reduced negative charge. Hence, the tendency to bind toxicants would be reduced. Cassity & Kolodziej (1984) suggest that binding of cations by extracellular polymers makes them more readily available for transport into the cell. It is possible that isolate 82Q responds to toxicants by producing a polymer which does not readily bind them. This may act as a barrier to their transport into the cell and therefore decrease their rate of action on the cell.

Thus, the two isolates 82Q and S3/A51, show distinct responses to copper and organotin with respect to their production of extracellular polysaccharide. This may reflect the differences observed in their abilities to tolerate the toxicants. Since isolate S3/A51 is more tolerant of copper than isolate 82Q, the production of a more negatively charged polymer, which will presumably have a greater ability to bind metals, appears to be a more successful mechanism. However, it should be noted that the mutant 82Q/15b, which produces a polymer with a reduced level of uronic acid, also shows a high tolerance to copper.

Tolerance to toxicants, like adhesiveness, might thus be dependent on structural properties of the polysaccharide rather than chemical composition. Possibly, tolerance might result from production of a polysaccharide with structural properties that result in exclusion of toxicants. Alternatively, the quantity of exopolysaccharide produced may influence the effect of the toxicants. No attempt was made in these studies to determine if

the presence of toxicants resulted in any change in the amounts of polysaccharide produced. The production of excessive amounts of exopolymer may afford protection against toxicants by acting as a diffusion barrier (Dempsey, 1981a,b) or as natural chelating material influencing the permeation of metals (Corpe, 1975).

### 3.6.2 Physical Properties

#### 3.6.2.1 Interaction with Ions

Polysaccharides as 1% (w/v) solutions for isolates 82Q and S3/A51 and 0.2% (w/v) solutions for isolate 82B were tested for their interactions with ions. Results are shown in Table 3.9. Few differences in ionic interactions were observed in polymers produced by isolates 82B and S3/A51 when grown in the presence and absence of toxicants. However, in the case of isolate 82Q, changes in the properties of the polymer produced were more obvious. In particular, in the presence of copper the polymer produced formed precipitates with a number of ions, whereas the polymer formed in the absence of copper showed no reaction with these ions. The polymer produced by the mutant 82Q/15b exhibited different types of interactions with ions compared to the parental strain, grown in the absence of toxicants. Notably, interaction with cobalt, copper and zinc ions resulted in the formation of precipitates rather than localized gels.

These differences in physical properties may result from the differences observed in chemical composition of the polymer. The

Table 3.9: Interactions of Extracellular Polysaccharides from Marine Isolates with Ions

| Isolate | Growth Medium | ASW | $Al^{3+}$ | $Ca^{2+}$ | $Co^{2+}$ | $Cu^{+}$ | $Cu^{2+}$ | $Fe^{2+}$ | $Fe^{3+}$ |
|---|---|---|---|---|---|---|---|---|---|
| 82Q | MIIYE | - | P | - | G | G | G | F | P |
| | MIIYE+0.1mM Cu | F | P | P | G | G | G | P | P |
| | MIIYE+0.1mM DMTC | - | P | - | G | G | G | P | P |
| 82Q/15b | MIIYE | - | F | - | P | P | P | P | O |
| 82B | MIIYE | - | P | - | P | P | P | P | - |
| | MIIYE+0.7mM Cu | - | P | - | O | - | O | O | - |
| | MIIYE+0.1mMDMTC | - | P | - | P | - | P | P | P |
| S3/A51 | MIIYE | - | - | - | - | - | - | G | G |
| | MIIYE+1mM Cu | - | G | - | - | P | - | G | G |
| | MIIYE+0.2mMDMTC | - | G | - | - | - | - | G | G |

-: no reaction; O: opaque solution; p: precipitate; f: flocculation; g: gel

Table 3.9: Interactions of Extracellular Polysaccharides from Marine Isolates with Ions(cont´d)

| Isolate | Growth Medium | $H^+$ | $K^+$ | $Mg^+$ | $Mn^{2+}$ | $Na^+$ | $Sr^+$ | $Zn^{2+}$ |
|---|---|---|---|---|---|---|---|---|
| 82Q | MIIYE | - | - | - | - | - | - | G |
| | MIIYE+0.1mM Cu | P | P | P | P | P | O | G |
| | MIIYE+0.1mM DMTC | - | - | - | - | - | - | G |
| 82Q/15b | MIIYE | - | - | - | - | - | - | P |
| 82B | MIIYE | - | - | - | - | - | - | P |
| | MIIYE+0.7mM Cu | - | - | - | - | - | - | P |
| | MIIYE+0.1mM DMTC | - | - | - | - | - | - | P |
| S3/A51 | MIIYE | - | - | - | - | - | - | F |
| | MIIYE+1mM Cu | - | - | - | - | - | - | F |
| | MIIYE+0.2mM DMTC | - | - | - | - | - | - | P |

most notable changes in the polysaccharide of isolate 82Q grown in the presence of copper, and that of mutant 82Q/15b was a reduction in the quantities of uronic acid. However, other workers have ascribed gel formation in the presence of cations to specific binding of uronic residues by these ions (Sutherland, 1980; 1982). If this mechanism also applied to other forms of interaction, a reduction in uronic acid content would be expected to lead to fewer interactions contrary to the results found in these studies. Nevertheless, the formation of precipitates rather than gels with some ions in the case of polysaccharide produced by mutant 82Q/15b might be attributed to the observed reduction in the the quantity of uronic acid present.

3.6.2.2 Viscosity

Polysaccharide solutions were examined to reveal their viscosity characteristics at different shear rates. Results are shown in figs.3.20-3.22. Most polysaccharides tested showed the pattern expected for a pseudoplastic polymer. Solutions of such polymers are subject to shear thinning, resulting in lower viscosities at high shear rates (Sutherland, 1982).

Polymer produced by isolate 82Q grown in the absence of toxicants appeared to be more viscous than that produced in the presence of DMTC, but less viscous than that produced in the presence of copper (fig.3.20). The mutant 82Q/15b produced a polymer with greatly reduced viscosity as compared to the parental strain when both were grown in the absence of toxicants. Changes

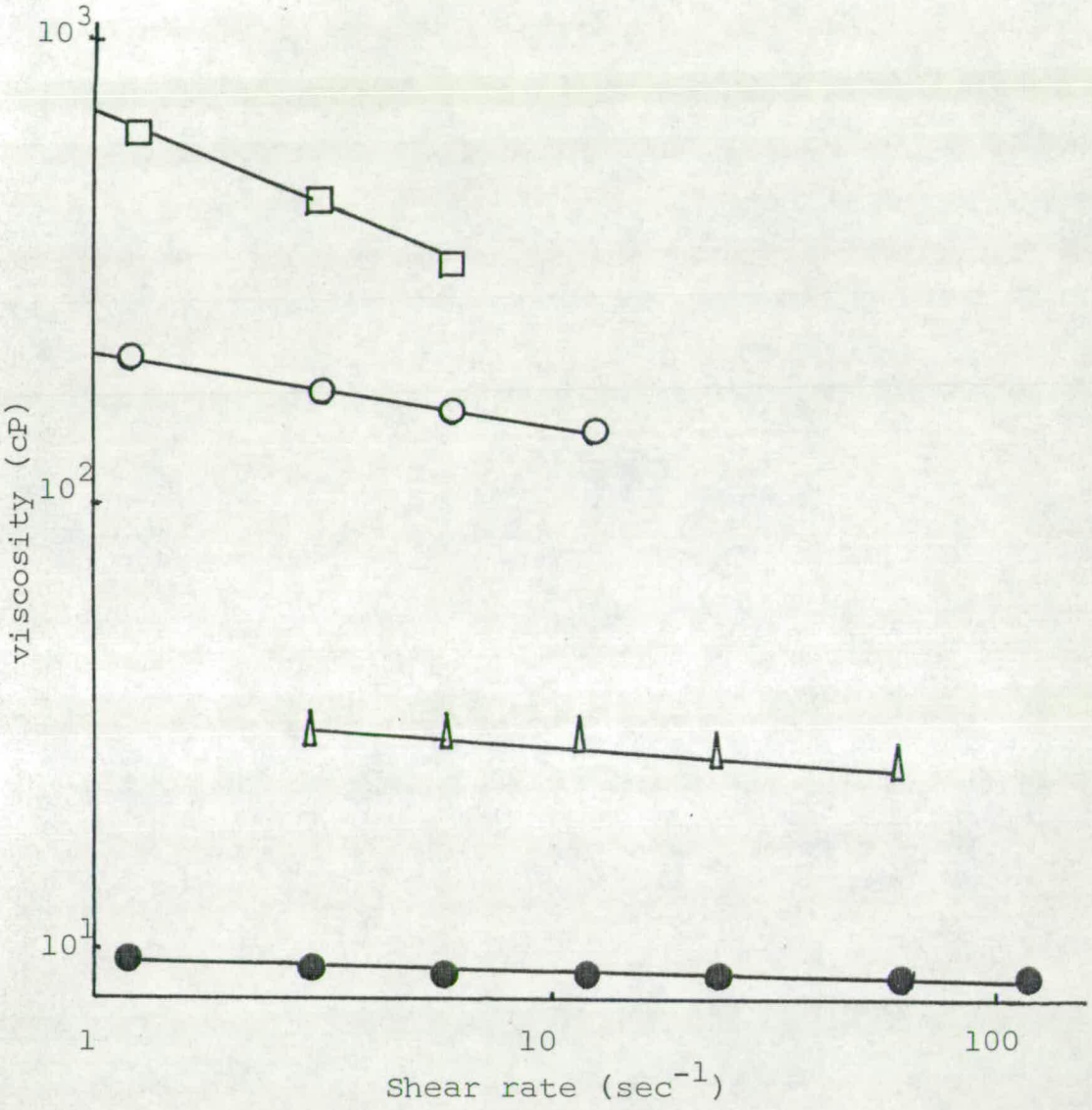



fig.3.20: Viscosity of extracellular polysaccharides (1% w/v) from isolate 82Q grown in MIIYE (○); MIIYE+0.1mM Cu (□); and MIIYE+0.1mM DMTC (△); and from mutant 82Q/15b grown in MIIYE (●).

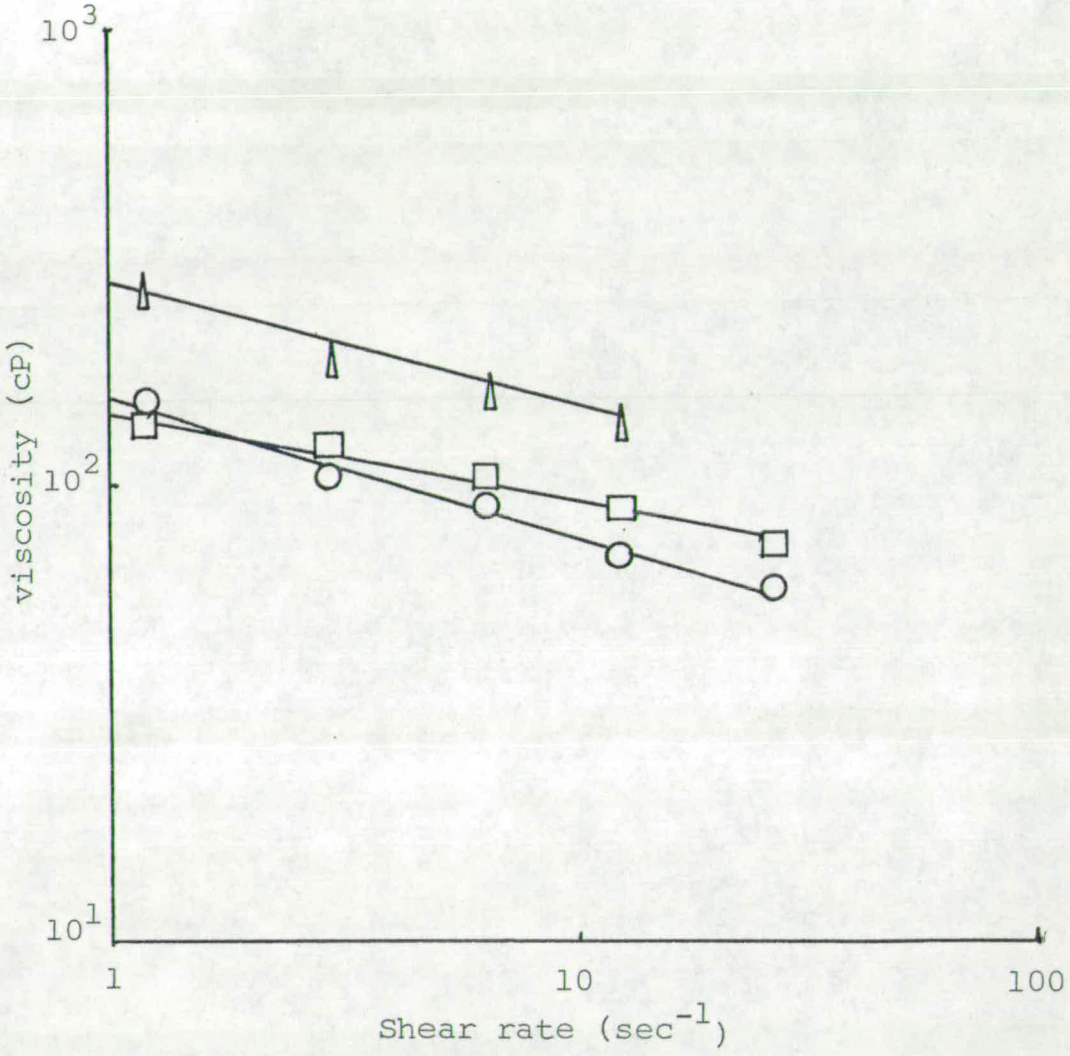



fig.3.21: Viscosity of extracellular polysaccharides (0.5% w/v) from isolate S3/A51 grown in MIIYE (○); MIIYE+1mM Cu (□); and MIIYE+0.2mM DMTC (△).

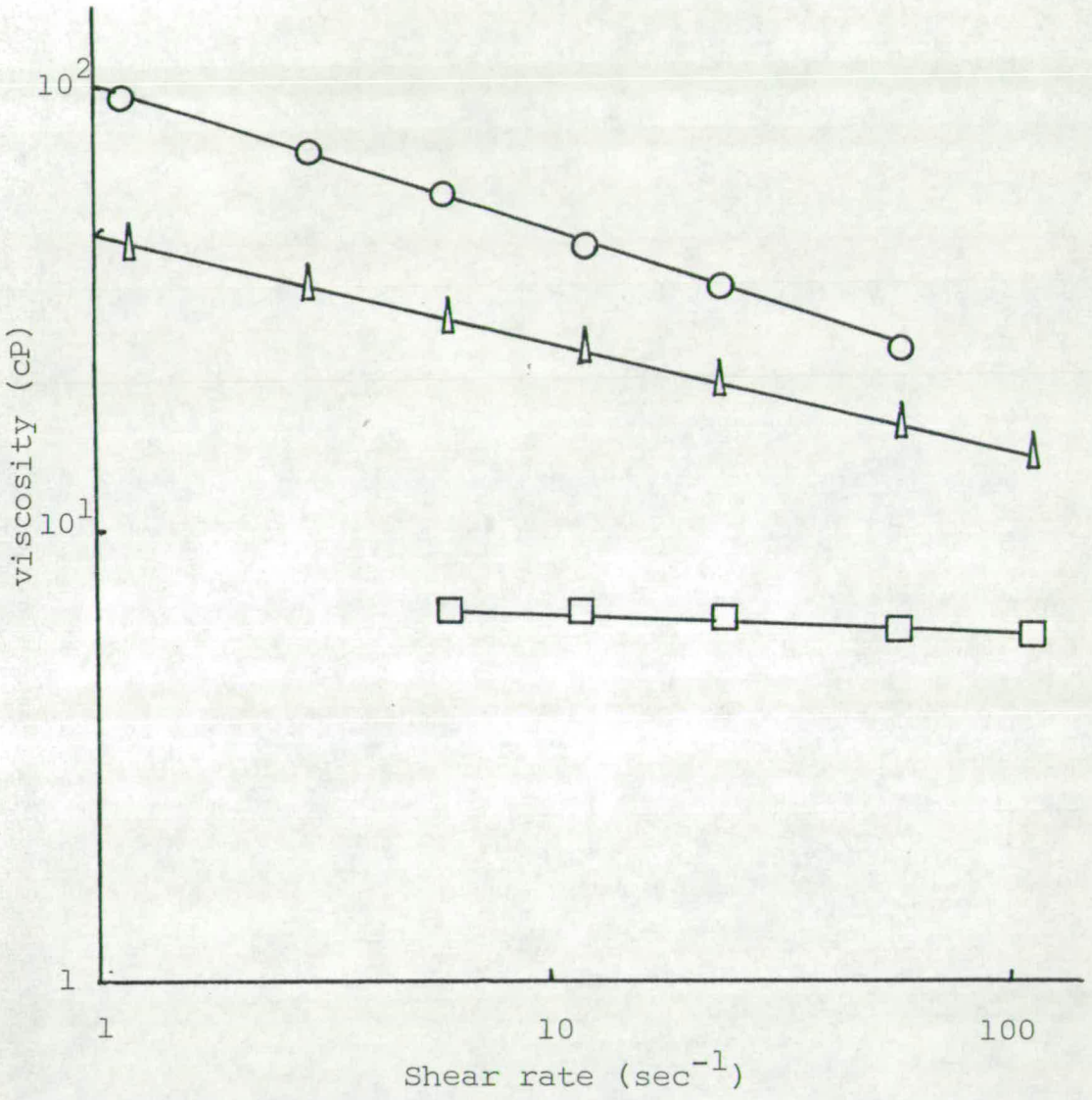



fig.3.22: Viscosity of extracellular polysaccharides (0.2%w/v) from isolate 82B grown in MIIYE (○); MIIYE+0.7mM Cu (□); and MIIYE+0.1mM DMTC (△).

were also observed in the pseudoplastic nature of the polymers.

Isolate S3/A51 exhibited less variation in the viscosity of polymers produced in the presence and absence of toxicants (fig.3.21). Those produced in the absence of toxicants and in the presence of copper showed similar properties, whereas the polymer produced in the presence of DMTC had increased viscosity.

Polymers produced by isolate 82B showed some variation in viscosity profiles (fig.3.22). In this case, the polymer produced in the absence of toxicants was most viscous and that produced in the presence of copper least viscous, with that produced in the presence of DMTC showing intermediate properties.

Factors influencing the viscosity of polymers include the shape, size and molecular mass of the polysaccharide molecule (Sutherland, 1982). The chemical nature of the bonds between monosaccharide residues, which affect rigidity of the molecule will also have an influence. In addition, the absence of acyl components appears to result in polymers of lower viscosities in some cases (Sutherland, 1983).

However, from results obtained in these studies, the variations in viscosity cannot be attributed to any particular differences in chemical composition of the polymers produced in the presence or absence of toxicants.

## 3.7 Effect of Toxicants on Proteins of Marine Bacteria

The binding of metal cations by bacterial extracellular polymers has been proposed to render them more available for transport into the cell (Cassity & Kolodziej, 1984). In Gram-negative bacteria, the outer membrane may act as an effective permeability barrier, and interactions between LPS and protein molecules are necessary for this function (Nikaido & Vaara, 1985). Major outer membrane proteins include the porins, which form hydrophilic channels and have a non-specific role in the passage of small molecules across the membrane. Minor proteins may serve as high affinity transport systems for nutrients or as receptors for bacteriophages and colicins. Metal ions are known to bind readily to sulphydryl groups exposed on the surface of proteins (Lonnerdal & Keen, 1982). Corpe (1975) suggests that heavy metal cations may interact with transport proteins and render them non-functional. In addition, binding of metals to outer membrane proteins might interfere with interactions between these proteins and LPS, thus affecting the barrier function of the outer membrane. Some outer membrane proteins may be implicated in attachment processes (Pringle *et al.*, 1983; Ward & Berkeley, 1980), thus binding of toxicants to such proteins may interfere with the tendency for attachment of cells to surfaces.

An outer membrane protein (H1) of *Pseudomonas aeruginosa* has been implicated in resistance to some antibiotics (Anwar *et al.*, 1983). Lutkenhaus (1977) reports that copper resistant mutants of *E. coli* lacked a major outer membrane protein thought to be involved

in the formation of hydrophilic pores. These reports suggest two possible strategies concerning outer membrane proteins in the development of tolerance to toxicants. Firstly, production of an outer membrane protein in the presence of toxicants may, in some manner, confer protection against the toxicant on the cell. Protein H1 of Pseudomonas aeruginosa is thought to confer resistance to polymyxin by protection of the sites of attack on LPS (Anwar et al., 1983). Several reports describe copper-inducible, low molecular weight proteins in either eukaryotes or prokaryotes, which may be able to mediate copper tolerance by sequestration of excess copper (Rouch et al., 1985). Secondly, inhibition of production of transport proteins in the presence of toxicants may prevent or reduce their uptake and thus their action on the cell.

In addition to outer membrane proteins, other bacterial proteins may be involved in resistance to toxicants. Rouch et al., 1985) propose that inducible, plasmid-mediated copper resistance in E. coli involves an intracellular binding protein, coupled with an efflux mechanism which ejects excess copper.

### 3.7.1 Effect on Outer Membrane Proteins

Initially, outer membrane fractions were prepared by two procedures, the Osborn method and the PBS-sarkosyl method (see Section 2.13). The method of Osborn et al. (1972) is widely accepted and has been used for a variety of purposes, including protein localization (Lloyd & Russell, 1984; Rodriguez-Tebar et al., 1985). However, in these studies the Osborn method yielded low

quantities of outer membrane material. This led to difficulties in detection of proteins by SDS-PAGE, even with use of the highly sensitive silver staining technique. Subsequently the PBS-sarkosyl method was employed in outer membrane preparation. Sodium lauryl sarcosinate (sarkosyl) has been shown to selectively disrupt the cytoplasmic membrane whilst the outer membrane remains intact (Filip et al., 1973). However, Rodriguez-Tebar et al. (1985) state that this method of membrane preparation appears to lead to differing conclusions on the subcellular localization of certain proteins. Chopra & Shales (1980) report that treatment with sarkosyl removed a number of high molecular weight polypeptides from the outer membranes of E. coli. Moreover, this treatment failed to remove residual inner membrane proteins as judged by the levels of NADH-dehydrogenase and succinic dehydrogenase. Anwar et al. (1983) suggest that this method should not be used in the preparation of outer membranes for the study of physical properties, and if used in chemical studies, caution should be exercised in interpretation of the results. However, in studies on outer membrane proteins of Azotobacter vinelandii, those prepared by the sarkosyl method were found to be almost identical to those prepared by the Osborn method (Page & Huyer, 1984). Since these studies involved only comparisons of the size of outer membrane proteins produced during growth in the absence and presence of toxicants, and all the samples were prepared by the sarkosyl procedure, this was an adequate method.

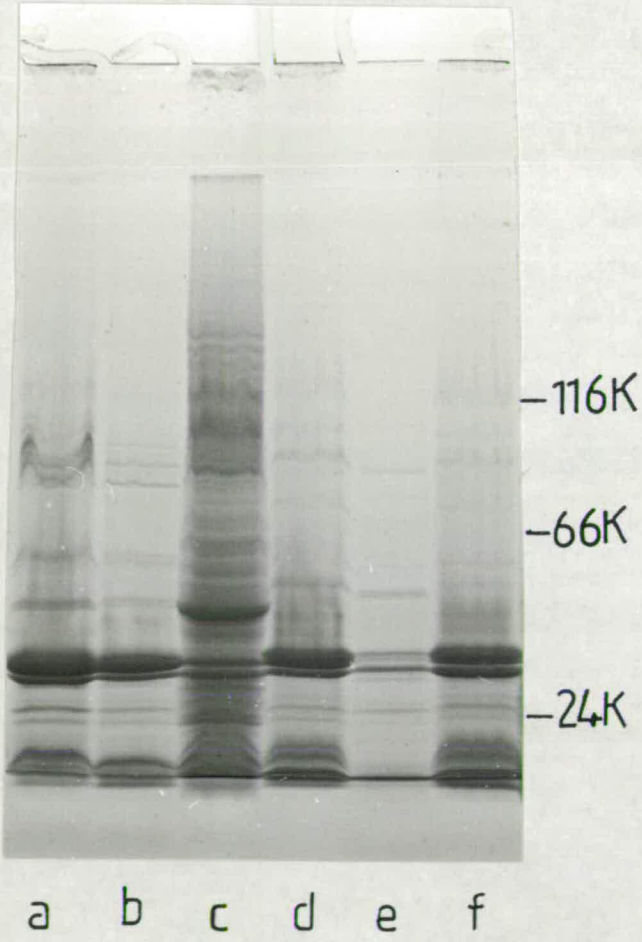
Outer membrane fractions from isolates 82B, 82Q and S3/A51 were analyzed by SDS-PAGE and detected by staining with PAGE blue 83 and

subsequent destaining. Results are shown in Plates 5-7. Outer membrane fractions are seen to contain a fairly large number of protein species. Nevertheless, these protein profiles are dominated by a low number of major protein bands. Early reports indicated that the outer membranes of Gram-negative bacteria contained just a few protein species, but the development of more sensitive methods of detection has revealed the presence of many minor proteins (Nikaido & Vaara, 1985). Characterization of outer membranes from Bacteroides species has revealed complex polypeptide patterns (Williams & Holt, 1985).

Outer membrane preparations from isolate 82B grown in the absence of toxicants, in the presence of 0.7mM copper sulphate and 0.1mM DMTC had the protein profiles shown in Plate 5. All three samples showed the presence of at least two major proteins, with relative molecular weights of 32K and 34K daltons. It is probable that these correspond to the porin proteins of similar molecular weight described in other species, which allow the passage of small hydrophilic molecules across the membrane (Nikaido & Vaara, 1985). In discussion of the permeability of porin channels, Nikaido & Vaara (1985) suggest the possibility of acquiring resistance by elimination of porins, since mutants which eliminate porins have been shown to have resistance to certain noxious agents. However, no changes were observed in the molecular weights of these porin-type proteins, thus it seems unlikely that this is the mechanism of tolerance in isolate 82B.

No obvious differences occur at all in the protein profile of outer membranes of isolate 82B grown in the presence of copper as

Plate 5: SDS-PAGE of outer membrane proteins of: isolate 82B produced during growth under routine conditions (a), in the presence of 0.7mM copper sulphate (b), and in the presence of 0.1mM DMTC (c); and isolate S3/A51 produced during growth under routine conditions (d), in the presence of 1mM copper sulphate (e), and in the presence of 0.2mM DMTC (f).
Position of molecular weight markers is shown.



compared to those grown in the absence of toxicants. However, during growth in the presence of DMTC some changes are apparent in the minor protein components. In particular, a number of high molecular weight proteins are produced in the range of 90-175K. Also, a protein band with a relative molecular weight of 45K stains more densely in this sample, and there may be some differences in low molecular weight proteins. These additional proteins which are induced in the presence of organotin may suggest a strategy whereby these proteins confer tolerance by protection against the toxicant, possibly by either sequestration of the toxicant or protection of its site of action.

The polypeptide profiles of outer membranes from isolate S3/A51 grown in the absence of toxicants, in the presence of 1mM copper sulphate and 0.2mM DMTC are also shown in Plate 5. Three major proteins with relative molecular weights of 33K, 35K and 37K are present in each sample, probably representing porin proteins. Thus, again tolerance does not appear to be related to elimination or substitution of major proteins involved in the uptake of toxicants. During growth in the presence of DMTC, no differences in the outer membrane proteins are apparent as compared to those present in the absence of toxicants. In contrast to isolate 82B, a few changes in the minor outer membrane proteins occur in the presence of copper. For instance, four protein bands of relative molecular weights 40K, 44K, 46K and 50K present in the sample grown in the absence of toxicants seem to be replaced in the presence of copper by two with relative molecular weights of 49K and 51K. These proteins may have some role in conferring resistance to copper

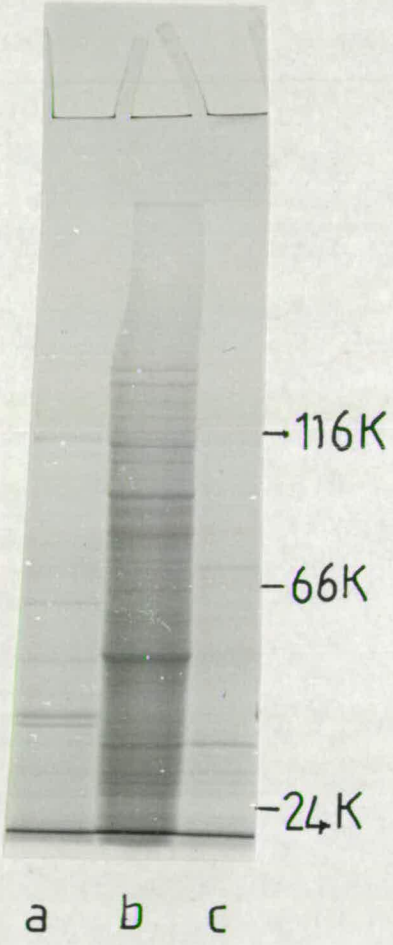
on isolate S3/A51. It is unlikely that outer membrane proteins are produced in sufficient quantities for sequestration of the relatively high concentrations of copper tolerated by isolate S3/A51. Thus, if these proteins are involved in tolerance mechanisms, the employment of some other strategy is probable. One possibility is that the altered proteins have an amino acid substitution on the surface exposed region of the structure such that they have a reduced tendency to bind toxicants. This might lead to lower quantities of toxicants being maintained near the surface for transport into the cell and subsequently to a reduction in their rate of reaction on the cell. Alternatively, some minor outer membrane proteins of Gram-negative bacteria are known to be involved in specific transport processes and may be inducible in the presence of specific groups of solutes (Nikaido & Vaara, 1985). An example of this is the glucose-inducible protein (D1) of Pseudomonas aeruginosa, as discussed by Nikaido & Vaara (1985). This protein is thought to form an ion-permeable channel with some specificity towards glucose. Minor outer membrane proteins in isolate S3/A51 may have similar properties, and might also be involved in the uptake of metal ions. Thus differences in the minor outer membrane proteins produced during growth in the presence of copper might be a reflection of changes in specific transport proteins leading to a decrease in the rate of copper uptake by the cells, and subsequently to increased tolerance.

Differences in the major outer membrane protein profiles of isolate 82Q were observed during growth in the presence of 0.1mM copper sulphate and 0.1mM DMTC as compared to those grown in the

absence of toxicants (Plate 6). Two major bands corresponding to proteins of relative molecular weights of 36K and 37K appear in the sample grown in the absence of toxicants. In the presence of copper or organotin, these proteins are absent or present in greatly reduced amounts. However, a densely staining band corresponding to a protein of relative molecular weight of 34K appears. This indicates a tolerance mechanism similar to that suggested by Lutkenhaus (1977) for copper tolerance in E. coli. Since tolerance to these toxicants leads to a loss of these major proteins, it is possible that the toxicants penetrate the outer membrane through pores formed by these proteins to reach a sensitive site. Since this isolate is still sensitive to high concentrations of both toxicants, it is unlikely that such proteins are actually the sites of action. Moreover, this suggests that toxicants may be able to penetrate other pores , although at a lower rate, indicating that this is not a greatly efficient strategy for tolerance.

Some changes were also observed in the minor outer membrane protein profiles. For instance, a protein with a relative molecular weight of 60K, present in the sample grown in the absence of toxicants, was not apparent in the other two samples. In addition, a band corresponding to a 47K protein stained with greater relative density in the sample grown in the presence of copper than in the other samples. Several other additional proteins were present in this sample, but caution must be taken in the interpretation of this result, since it may reflect a more concentrated sample with respect to protein content. However, changes in minor outer membrane proteins of isolate 82Q may be a

Plate 6: SDS-PAGE of outer membrane proteins of isolate 82Q produced during growth under routine conditions (a), in the presence of 0.1mM copper sulphate (b), and in the presence of 0.1mM DMTC (c). Position of molecular weight markers is shown.



strategy in tolerance to toxicants, in the same manner as that described above for isolate S3/A51.

Mutant 82Q/15b, which is more resistant to higher concentrations of copper than the parental strain, shows differences in the outer membrane protein profile as compared to the parental strain when both are grown in the absence of toxicants (Plate 7). The most notable difference is the absence of the 36K and 37K proteins found in the parental strain. These appear to be replaced in the mutant by three major bands representing proteins with relative molecular weights of 26K, 29K and 31K. This again indicates a tolerance mechanism involving the control of uptake of toxicants by the cell. During growth of mutant 82Q/15b in the presence of 0.8mM copper sulphate, the 29K and 31K proteins appear to be repressed, and several minor proteins appear to be induced. In particular, additional proteins with relative molecular weights of 50K, 96K and 113K were apparent. These may in some way be involved in conferring resistance to copper on the mutant.

In these studies, only the molecular weight of the outer membrane proteins was determined. Although, in the case of isolates 82B and S3/A51, no changes in the molecular weights of the major outer membrane proteins occurred during growth in the presence of toxicants, this is not conclusive evidence that there is no change in their tendency to bind copper. Copper is known to bind strongly to the amino acids cysteine, histidine and tryptophan (Lonnerdal & Keen, 1982). Thus substitution of one of these residues for an amino acid with less tendency to bind copper, which may not result in a detectable change in the molecular weight of the

protein, could provide a possible strategy for copper tolerance.

The outer membrane fractions used in this work were prepared from cells grown at 30° C. However, the temperature of growth may affect the production of outer membrane proteins, and thus different protein profiles might be observed in the natural marine environment. Nevertheless, these results do indicate the ability of marine isolates to alter outer membrane proteins in response to the presence of divalent copper ions or organotin.

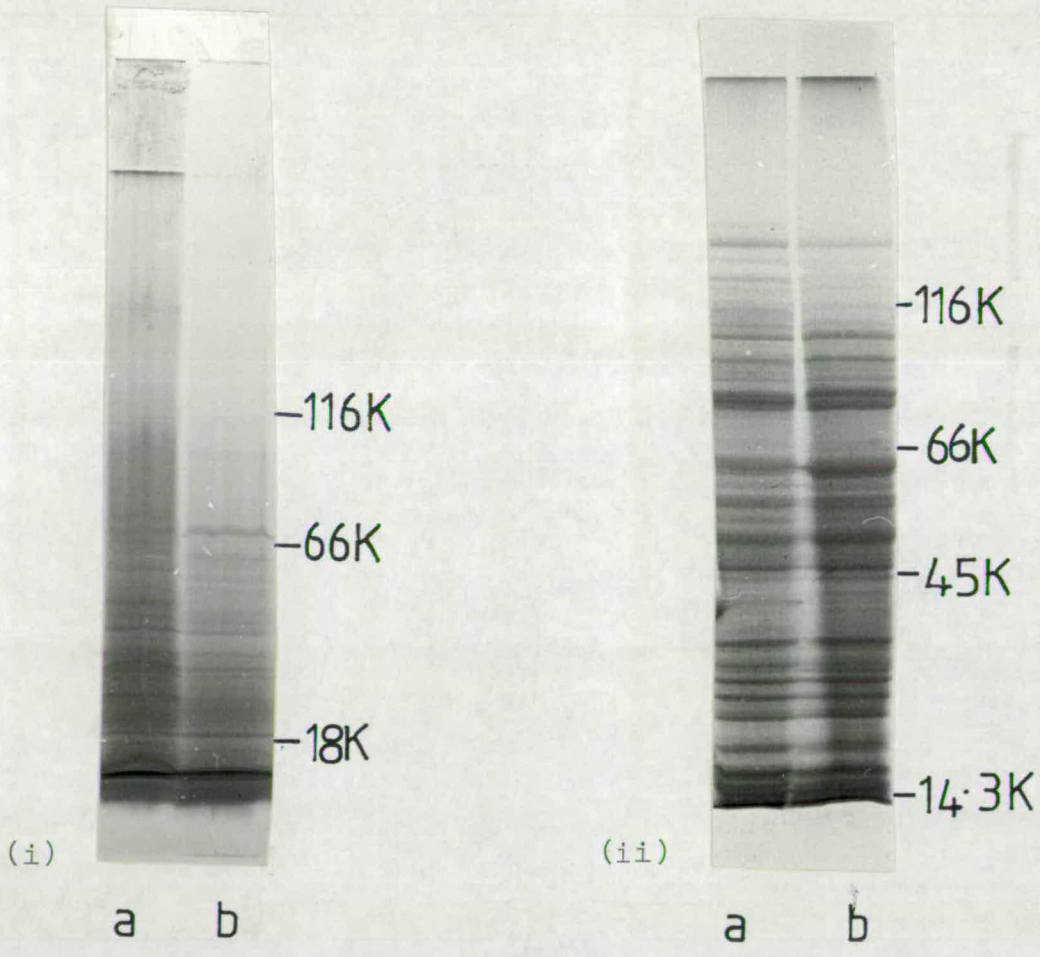
In addition to outer membrane proteins, LPS is also known to bind metal ions (Heptinstall et al., 1970; Strain et al., 1983). Divalent cations dramatically affect the melting temperature of acidic phospholipids, and the nature of the cations interacting with LPS is important (Nikaido & Vaara, 1985). Thus, binding of divalent copper ions to LPS may competitively inhibit the binding of other essential ions resulting in disruption of the membrane. Alterations in the LPS component of the outer membrane might provide another possibility for the development of tolerance to metal ions.

### 3.7.2 Effect on Cytoplasmic and Periplasmic Proteins

Soluble cell free extracts of isolate S3/A51 and mutant 82Q/15b retained during the preparation of outer membrane fractions by the PBS-sarkosyl method (see Section 2.14) were analyzed by SDS-PAGE. Gels were stained by the PAGE blue 83 method. These samples would be expected to contain both cytoplasmic and periplasmic proteins. Results are shown in Plate 8. With both isolates, it was observed

Plate 8: SDS-PAGE of cytoplasmic and periplasmic proteins produced by: isolate S3/A51 (i) during growth under routine conditions (a), and in the presence of 1mM copper sulphate (b); and mutant 82Q/15b (ii) during growth under routine conditions (a), and in the presence of 0.8mM copper sulphate (b).

Position of molecular weight markers is shown.



that some proteins detected in these fractions were also present in the outer membrane fractions. This might be due to incomplete separation of the two fractions, or might suggest that such proteins are loosely bound to the outer membrane and are partly released during breakage and centrifugation.

Isolate S3/A5l showed few changes in the cytoplasmic and periplasmic proteins produced during growth in the presence of 1.0mM copper sulphate compared to those produced under routine growth conditions (Plate 8a). In the presence of copper, it appeared that proteins with relative molecular weights of 26K and 46K were repressed and a 59K protein was induced. In the presence of 0.8mM copper sulphate, some cytoplasmic and periplasmic proteins of mutant 82Q/15b produced under routine growth conditions seemed to be repressed (Plate 8b). These included high molecular weight proteins of 132K, 150K and 162K, and a 39K protein. Rouch et al. (1985) propose the involvement of an intracellular binding protein in copper tolerance to E. coli. No indication of such a mechanism has been found in the examination of cytoplasmic and periplasmic proteins of two isolates in these studies. However, changes in the protein profiles of tolerant bacteria when grown in the presence of copper would be expected only if tolerance was inducible. It has already been indicated that, in the case of isolate S3/A5l, tolerance may be constitutive since non-growing cells exhibit greater tolerance than growing cells (see Section 3.4.2). Thus, if tolerance is related to bacterial proteins, the proteins involved would be expected to be present during growth in either the absence or the presence of copper. Any changes in the protein profiles

observed in the presence of copper might therefore be due to a coincidental effect of the toxicant on cell processes not involved in tolerance.

## 3.8 Immobilized Metal Affinity Chromatography of Outer Membrane Fractions

Marine isolate S3/A51 has shown properties which suggest that its tolerance to divalent copper ions may be constitutive rather than inducible. Thus if the mechanism of tolerance involves outer membrane proteins, such proteins would be produced during growth in the absence as well as in the presence of copper, and might have copper-binding properties. Determination of copper-binding properties of outer membrane proteins may clarify their possible role in tolerance to copper.

Immobilized metal affinity chromatography (IMAC) has been used as a purification technique for proteins such as human plasma proteins (Moroux et al., 1985), and a number of other proteins including cytochrome c, lysozymes, Staphylococcal protein A and interferons (Sulkowski, 1985). The development of affinity chromatography was based on the concept of two molecules having biological affinity toward each other, and that this property can be used to separate one of these molecules from a complex mixture (Lonnerdal & Keen, 1982). In IMAC, the underlying event leading to adsorption is coordination between an immobilized metal ion, such as divalent zinc or copper ions, and an electron donor (Sulkowski, 1985). The metal ion is immobilized (usually chelated) on an

insoluble matrix support. The incoming protein binds to the metal ion via electron donor groupings resident on its molecular surface. Thus, amino acid residues with electron-donating side chains such as histidine, cysteine and tryptophan (Lonnerdal & Keen, 1982), when exposed on the protein surface will confer metal binding properties on the protein. Adsorbed proteins can subsequently be eluted from the column by a decrease in pH or by addition of EDTA to the elution buffer (Lonnerdal & Keen, 1982). This technique may be useful in examination of copper-binding properties of bacterial outer membrane proteins.

### 3.8.1 Intact Outer Membrane Fractions

Outer membranes from isolate S3/A51 grown in the presence of 1.0mM copper sulphate were prepared by the PBS-sarkosyl method, resuspended in starting buffer (see Section 2.16) and examined by IMAC to detect the existence of copper-binding properties. The elution of proteins was followed by measurement of the $E_{280}$ of fractions (fig.3.23). Peak (a) represents the non-specific proteins in the sample, which did not bind to copper. The larger peak (b) represents the copper-binding fractions of the outer membrane and indicates that most of the material had been bound to copper. SDS-PAGE of the pooled material from each of the peaks confirmed this observation (Plate 9). Silver staining was required to detect the small quantities of protein eluted from the column. Very few proteins were detected in the non-specific fraction. This would be expected since the membranes were not solubilized and thus binding

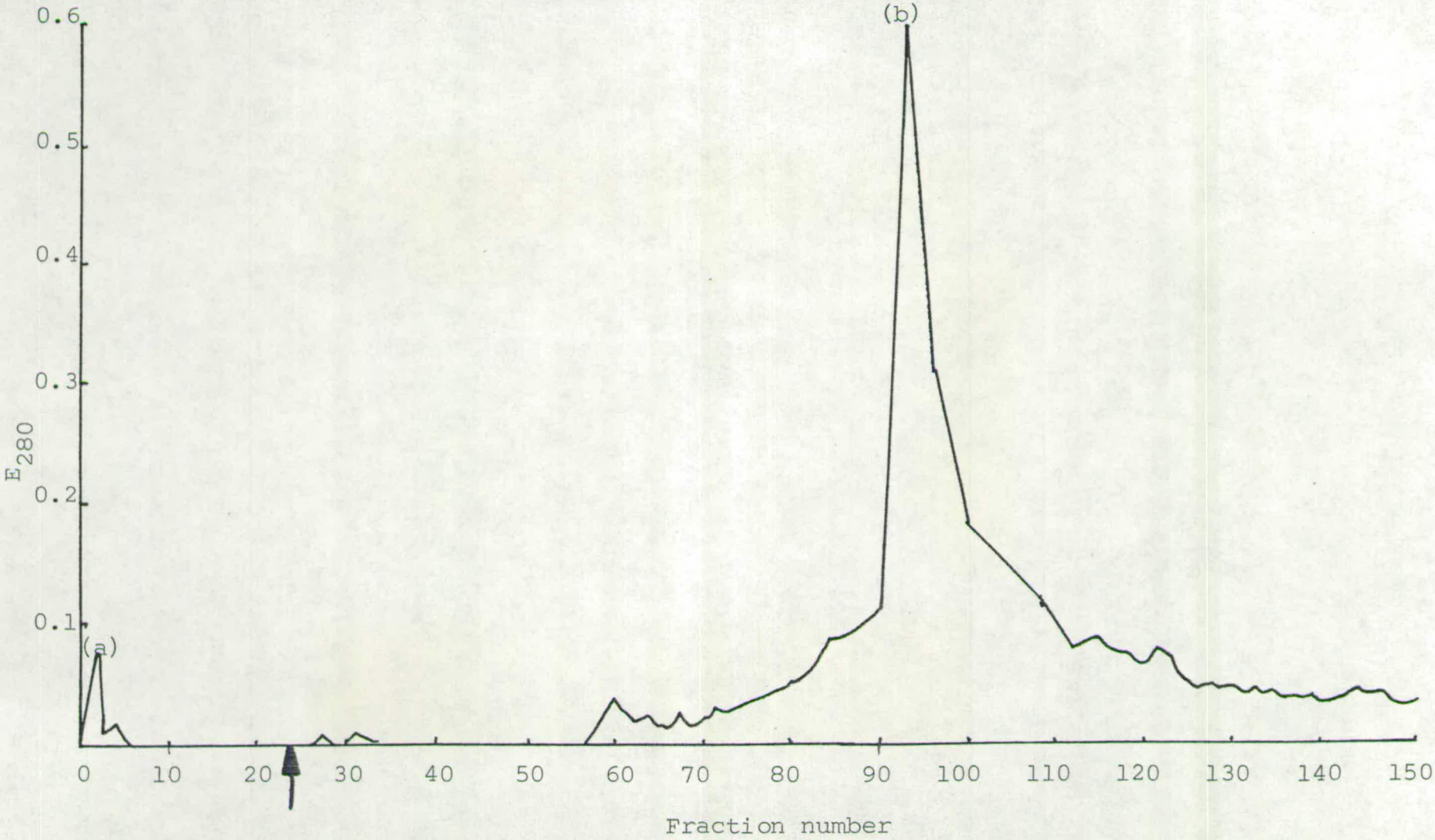



fig.3.23: Immobilized metal affinity chromatography of intact outer membrane fractions from isolate S3/A51 grown in the presence of 1mM copper sulphate. Arrow represents application of pH gradient.

Plate 9: SDS-PAGE of protein fractions obtained by IMAC of intact outer membranes of isolate S3/A51 grown in the presence of 1mM copper sulphate.

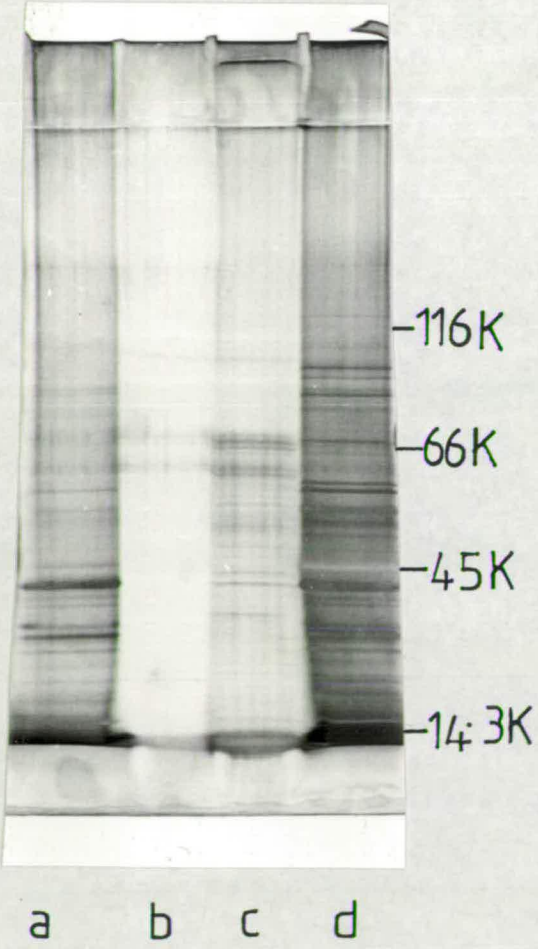
(a) outer membrane proteins of S3/A51 produced during growth in the presence of 1mM copper sulphate.

(b) non-specific proteins.

(c) copper-binding proteins.

(d) outer membrane proteins of S3/A51 produced during growth under routine conditions.

Position of molecular weight markers is shown.



to copper by any of the proteins would have the effect of retaining most of the material on the column. The few proteins which were observed in the non-specific fraction were also present in the copper-binding fraction, suggesting that these may be loosely bound outer membrane proteins, partly released in the preparation procedures.

These results give no indication of the copper-binding properties of any individual outer membrane proteins of isolate S3/A51. Nevertheless, the ability of outer membranes of this isolate to bind copper ions is demonstrated, and confirms their involvement in interaction with toxicants. Binding of metals to outer membranes of E. coli has been demonstrated by other techniques including phosphorous nuclear magnetic resonance (Ferris & Beveridge, 1984) and electron microscopy (Hoyle & Beveridge, 1983). Observations from such studies seem to suggest that LPS contains the high affinity binding site for metals (see Section 1.8.1). However, Beveridge & Koval (1981) suggest that metal ions may also interact with acidic groups of exposed outer membrane proteins.

### 3.8.2 Solubilized Outer Membrane Proteins

In order to determine copper-binding properties of individual proteins, it was necessary that the outer membranes were denatured. Porath & Olin (1983) report that ionic detergents such as SDS, at concentrations above 0.1%, virtually abolished the adsorption of proteins on immobilized metal affinity columns. However, uncharged detergents such as Tween 80 did not influence adsorption at low

concentrations, and at concentrations greater than 1% the adsorption capacity was increased. Thus, in these experiments outer membrane proteins were solubilized in starting buffer containing 1% Tween 80 (see Section 2.16).

Solubilized outer membrane proteins from isolate S3/A51 grown in the absence and presence of 1.0mM copper sulphate were examined for copper-binding properties by IMAC. The elution profile of protein obtained by $E_{280}$ measurement of the sample produced in the presence of copper (fig.3.24) indicates that most protein was eluted in peak (a) which represents the non-binding proteins. Copper-binding proteins were eluted by a gradient reduction in pH, and are represented by peak (b). However, no distinct peak in the $E_{280}$ profile was observed over the pH gradient, thus buffer containing 0.05M EDTA was used to elute any remaining adsorbed material.

SDS-PAGE of pooled material from peak (a), peak (b) and the material eluted with EDTA confirmed that most of the proteins present did not bind to copper (Plate 10). Few bands were observed in the copper-binding fractions, and the proteins which did appear were also present in the non-specific fraction. This would indicate that such proteins only bind copper weakly, and gives no conclusive evidence that any outer membrane proteins are involved in interaction with, or tolerance to, divalent copper ions.

Determination of the primary structure of outer membrane proteins of E. coli, including protein I (porin) (Chen et al., 1982), protein II (Chen et al., 1980) and lipoprotein (Braun et al., 1976) has revealed that the number of residues with

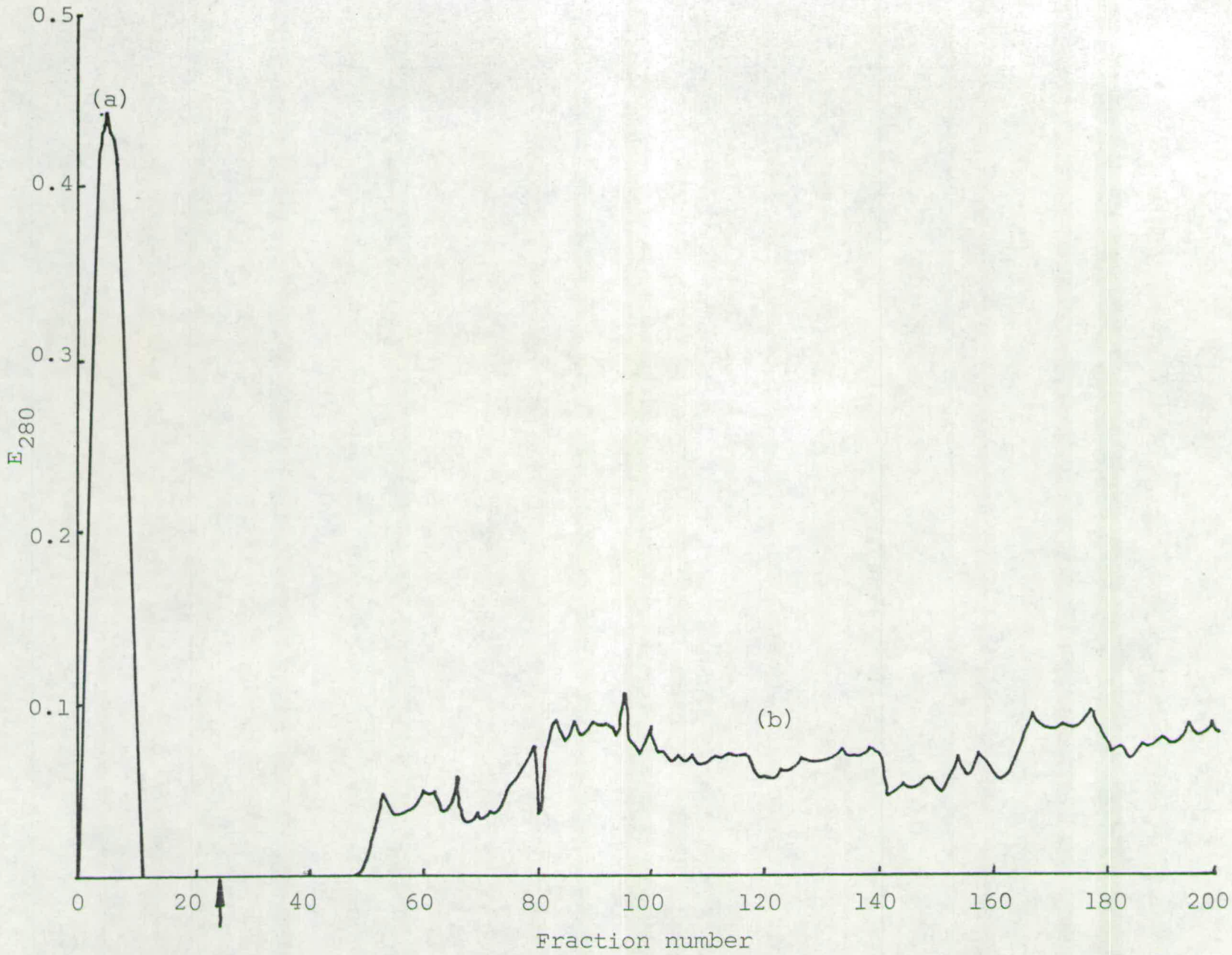



fig.3.24: Immobilized metal affinity chromatography of solubilized outer membrane fractions from isolate S3/A51 grown in the presence of 1mM copper sulphate. Arrow represents application of pH gradient.

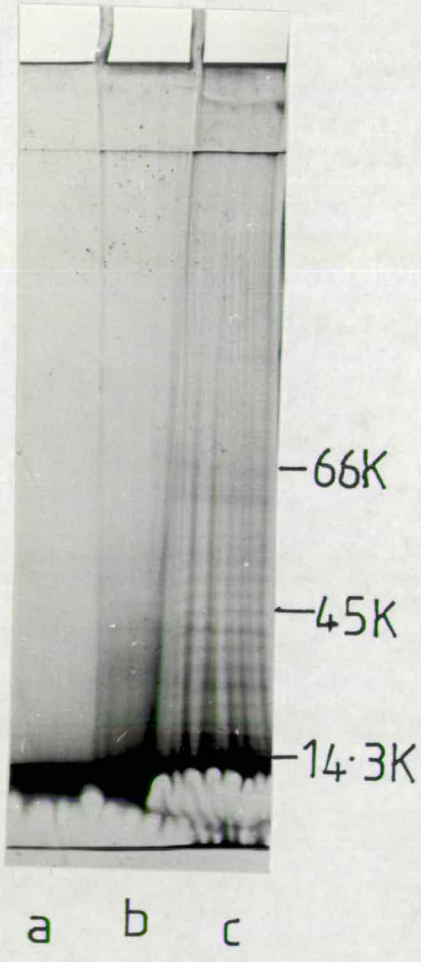
Plate 10: SDS-PAGE of protein fractions obtained by IMAC of soluble outer membranes of S3/A51 grown in the presence of 1mM copper sulphate.

(a) material eluted with EDTA.

(b) copper-binding proteins eluted by gradient reduction in pH.

(c) non-specific proteins.

Position of molecular weight markers is shown.



electron-donating side chains, such as histidine, cysteine and tryptophan, is very low. If the major outer membrane proteins of isolate S3/A51 possessed similar properties, it would be expected that these proteins at least would not bind strongly to copper.

Outer membranes from isolate S3/A51 grown in the absence of copper, treated similarly and examined by IMAC yielded contrasting results. The elution profile (fig.3.25) indicated that the majority of proteins were adsorbed on the column, and relatively little material was eluted in the non-specific fractions. This was confirmed by analysis of the material from each peak by SDS-PAGE. However, these results may reflect incomplete solubilization of the membrane, as they show similarities to the results observed above in examination of complete outer membranes.

Nevertheless, SDS-PAGE of the samples did reveal some information. One band representing a protein with a relative molecular weight in the 50K region observed in the copper-binding fraction did not appear in the non-specific fraction (Plate 11). Moreover, in comparison of whole outer membrane samples run on the same gel, this protein was present in the sample prepared in the absence of copper, but was absent in the sample prepared in the presence of copper. This copper-binding protein may be involved in some way in the uptake of copper by the cell. Thus, these results might indicate that the apparent repression of this protein during growth in the presence of copper is implicated as a strategy for tolerance. Such a strategy would entail an inducible mechanism which is contrary to previous evidence that tolerance may be constitutive (see Section 3.4.2). However, this evidence was based

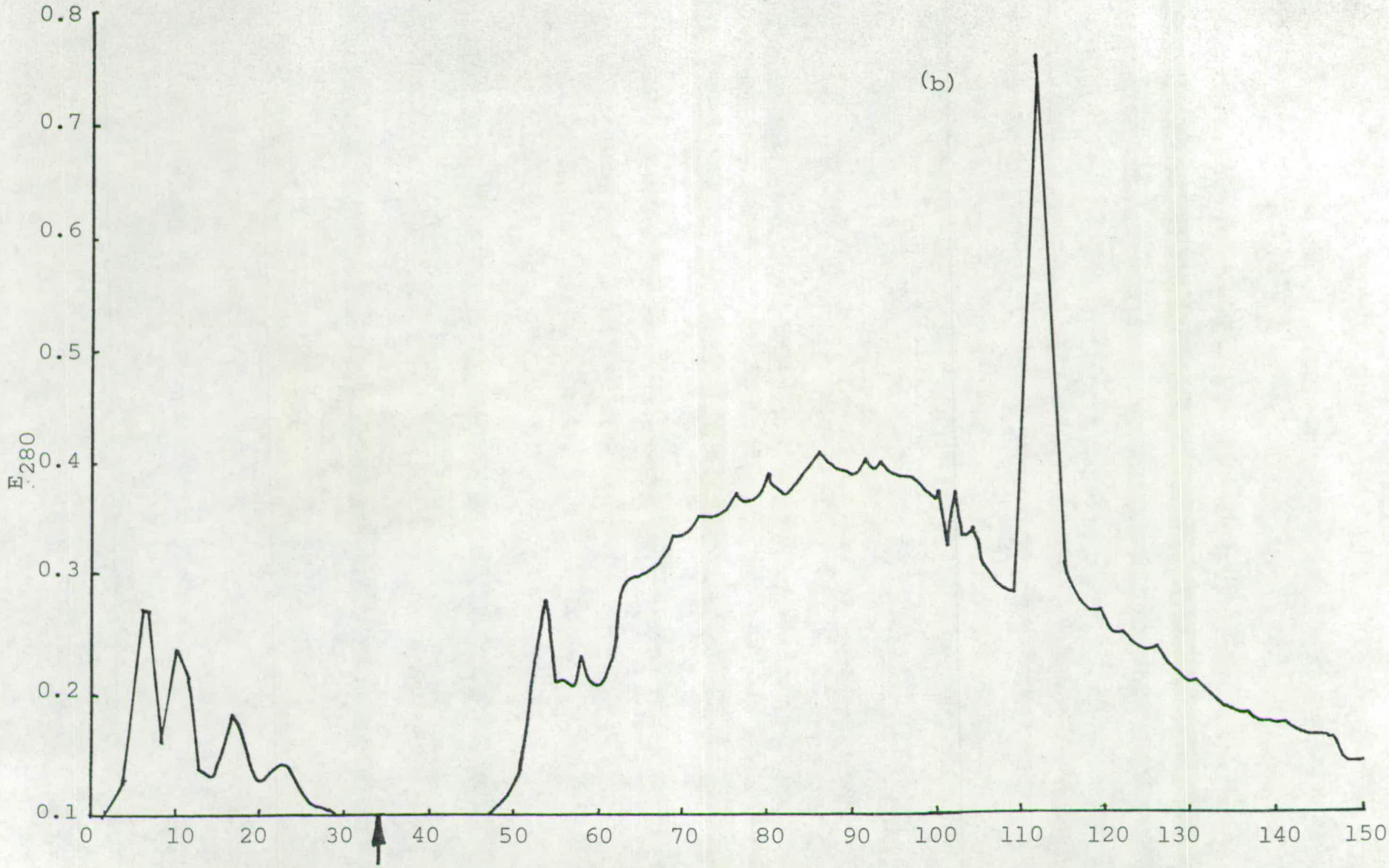



fig.3.25: Immobilized metal affinity chromatography of solubilized outer membrane fractions from isolate S3/A51 grown under routine conditions. Arrow represents application of pH gradient.

Plate 11: SDS-PAGE of protein fractions obtained by IMAC of soluble outer membranes of S3/A51 grown under routine conditions.

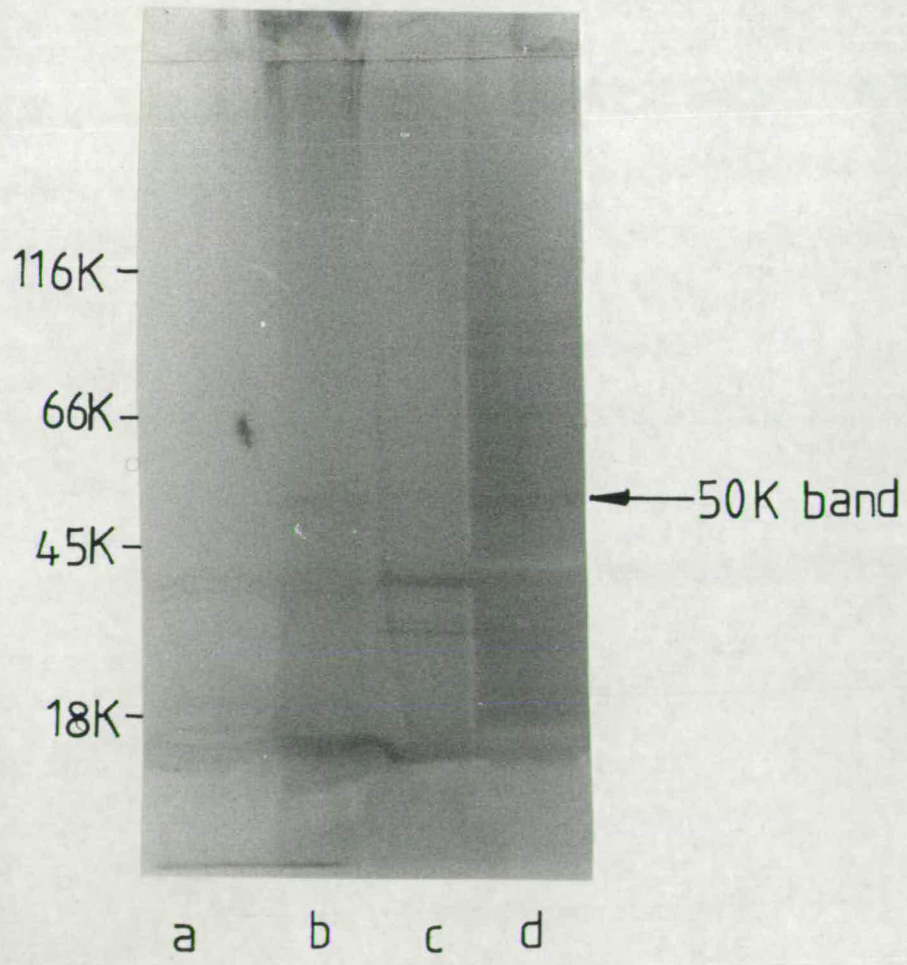
(a) non-specific proteins.

(b) copper-binding proteins.

(c) outer membrane proteins of S3/A51 produced during growth in the presence of 1mM copper sulphate.

(d) outer membrane proteins of S3/A51 produced during growth under routine conditions.

Position of molecular weight markers is shown.



on the observation that non-growing cells of isolate S3/A51 were more resistant to copper than growing cells. Another interpretation of this phenomenon might be that the increased resistance of non-growing cells is due to their failure to transport copper into the cell rather than to constitutive tolerance. Previous analysis of whole outer membrane samples of isolate S3/A51 (see Section 3.7.1) showed changes in minor outer membrane proteins, including some proteins with molecular weights in the region of 50K, during growth in the presence of copper. This may also indicate an inducible tolerance mechanism.

These results must be treated with caution, since membranes were denatured by treatment with detergent. Although this was necessary to allow the proteins to exhibit their individual copper-binding properties, it has the disadvantage that the whole protein surface is made available for interaction with copper rather than only the region normally exposed at the surface of the membrane. Thus amino acid residues, such as histidine, cysteine and tryptophan, which might normally be involved in interactions with LPS and cations maintaining the integrity of the membrane, may be exposed and allow adsorption of the protein on the column. However, outer membranes do have different affinities for different cations (Nikaido & Vaara, 1985). Copper ions may be able to displace other ions, such as calcium and magnesium ions, from the membrane and interact with membrane components, including proteins, in such a way as to disrupt the integrity of the membrane. Therefore, it might be argued that consideration of the whole protein surface is relevant.

# CHAPTER FOUR: GENERAL DISCUSSION

## 4.1 Introduction

It is clear that studies on the interaction of copper and organotin with marine bacteria are complicated by the differing responses of individual species to these toxicants. Previous work has dealt mainly with copper binding and tolerance in *E. coli*, which may be of little relevance to the responses of typical marine bacteria. It has been suggested that marine bacteria show greater resistance to heavy metal attack than terrestrial bacteria, possibly due to differences in cell wall and membrane structures and their various interactions (Arcuri & Ehrlich, 1977). Marine bacteria used in studies concerning copper-binding properties include pseudomonads (Corpe, 1975), and determination of copper toxicity has been carried out on a marine vibrio (Schreiber *et al.*, 1984) and on a range of unidentified, Gram-negative marine isolates (Arcuri & Ehrlich, 1977). However, little information is available on the binding of, mode of action of, or tolerance to organotin compounds in marine bacteria.

The work presented here indicates the diversity of marine bacterial populations, and the differing responses of such populations to toxicants. It appears that the toxicants present in antifouling paints select for a specific microbial fouling population, as indicated in previous studies by Dempsey (1981a,b).

In contrast to the information available on the response of marine bacteria to toxicants, the quantity of literature available on attachment of marine bacteria to surfaces is vast. However, little work has been carried out on the effects of the toxicants

used in antifouling paints on attachment, apart from the studies by Dempsey (1981a,b) determining the successional development of microbial biofilms on these paints. The toxicants appear to deter or retard attachment of marine bacteria, but the process by which this occurs is unclear. There may exist in the natural marine environment a range of bacteria which have the inherent ability to tolerate copper or organotin, or alternatively the presence of these toxicants may induce some bacteria to respond in some way that confers tolerance on the cell. Either way, it is not clear which properties are responsible for bacterial resistance to copper or organotin. Corpe (1975) suggests that marine bacteria may produce natural chelators which could influence the permeation of heavy metals, and Arcuri & Ehrlich (1977) discuss the possibility that tolerance may be due to cell wall or membrane structures. In addition, Dempsey (1981a,b) reports that excessive polysaccharide production is likely to provide some form of protection from toxic effects.

## 4.2 Action of Toxicants on Marine Bacteria

The growth of marine isolates in the presence of a range of concentrations of copper showed their variation in ability to tolerate copper. Isolates S3/A51 and 82B showed greatest tolerance, though at higher concentrations morphological changes and loss of motility in the cells was observed. The low numbers of cells of isolate 82Q surviving at higher concentrations of copper also showed a change in morphology and a loss of motility. These

observations suggested that copper has a detrimental effect on the process of cell wall synthesis, since the bacterial cell wall is responsible for the maintenance of cell shape. This effect might be caused by binding to and inhibiting enzymes involved in synthesis, or by competitively inhibiting the uptake of ions essential for cell wall synthesis or maintenance of the cell wall structure.

Further studies on isolate S3/A51 also suggested some effect on the process of cell division, since some cells appeared by SEM to be unable to undergo division. The ability of copper to bind to whole outer membranes of isolate S3/A51 has been demonstrated by IMAC. It is possible that the interaction of copper ions with the outer membrane may disrupt the membrane structure and destroy or reduce its action as a permeability barrier. In addition, an effect of copper on biosynthetic pathways was implied since there were differences in the ratios of monosaccharide constituents and quantities of acyl components of extracellular polysaccharides, and in the relative molecular weights of cellular proteins produced in the isolates examined, during growth in the presence of copper.

Marine isolates also exhibited variation in their ability to tolerate organotin during growth in the presence of a range of concentrations of DMTC. In contrast to the action of copper, no morphological changes were observed , suggesting that the site of action of DMTC in marine bacteria may differ from that of copper. However some action on biosynthetic pathways is probable, since changes were again observed in polysaccharides and proteins produced during growth in the presence of DMTC.

## 4.3 Effect of Toxicants on Attachment of Marine Bacteria

In the two marine isolates 82Q and S3/A51, differences were found in the effects of toxicants on cell survival and attachment to glass surfaces. Both survival and attachment of isolate 82Q were reduced as the concentration of copper sulphate or DMTC increased, implying that inhibition of attachment may be related to loss of cell viability. In the case of isolate S3/A51 however, as the concentration of either copper or organotin increased, attachment decreased without any loss of cell viability. This suggested that the reduction in attachment may have been caused by interactions of the toxicants with cell surface components involved in attachment mechanisms. The decrease in attachment only occurred between the control and the lowest concentrations of toxicants. As the concentration was increased, no further reduction in viability of attached cells was observed, indicating that prevention of attachment by the toxicants was less effective on isolate S3/A51 than on isolate 82Q. Mutant 82Q/15b showed a similar response to copper when the cells had previously been grown in the presence of copper. When grown in the absence of copper though, the cells showed a similar response to the parental strain 82Q.

Cell surface components proposed to have a role in the attachment process include extracellular polysaccharide and possibly some minor outer membrane proteins. The cells used in attachment studies on isolates 82Q and S3/A51 had previously been grown in the absence of toxicants. Thus, the properties of polysaccharides and proteins produced under such growth conditions would be important in

determining interactions with toxicants.

Comparison of the extracellular polysaccharides of isolates 82Q and S3/A5l revealed that, although the two polymers appeared to contain the same monosaccharide constituents, these were present in different molar ratios (see Section 3.6.1). Notably, polymer from isolate S3/A5l contained a higher ratio of fucose residues. Since differences in the ratio of monosaccharide constituents can imply differences in structure, and polysaccharide structure rather than chemical composition is thought to be responsible for adherence properties (Sutherland, 1980; 1982), this difference in ratios may be responsible for variation in attachment tendencies. In addition, differences were found in the acyl components of the two polymers and in their interactions with cations. Polymer produced by isolate 82Q had a higher uronic acid content, a lower content of acetate and pyruvate, and showed a greater tendency to interact with ions than that of isolate S3/A5l. The greater tendency to interact with ions, particularly resulting in gel formation may be related to the higher uronic acid content of polymer from isolate 82Q, since it has been proposed that, in some polymers, gel formation in the presence of cations is due to specific binding of uronic acid residues by these ions (Sutherland, 1980; 1982). Moreover, since this polymer underwent gel formation in the presence of copper ions, in contrast to that of isolate S3/A5l, this reaction could possibly be responsible for the greater reduction in attachment.

The extracellular polymer produced by mutant 82Q/15b contained the same monosaccharide constituents and showed little difference in the acetate and uronic acid composition when grown in the presence

or absence of copper. Insufficient quantities of the polymers were obtained to allow further comparisons, which might have revealed differences in the structural or physical properties of the polymers. Such properties might have been responsible for the decreased tendency of cells of the mutant, previously grown in the absence of copper, to attach when allowed to interact with copper ions. Comparison of the outer membrane proteins of this mutant revealed some differences in the proteins produced during growth in the absence and presence of copper (see Section 3.7.1). Outer membrane proteins are presumed to be available to the external environment, but their role in attachment is uncertain (Ward & Berkeley, 1980). Thus, though these changes in outer membrane proteins may be involved in some way in the difference in attachment properties after growth of the mutant in the presence of copper, no conclusive evidence as to their role in the attachment process has emerged.

## 4.4 Tolerance of Marine Bacteria to Toxicants

The attempt to determine the mechanism of tolerance to toxicants in the three marine isolates 82B, 82Q and S3/A51 revealed that bacteria may adopt various strategies to resist the toxic action. In isolate S3/A51 and mutant 82Q/15b, tolerance to copper appeared to be stably inherited, suggesting that this property may be chromosomally-encoded. In addition, particularly in isolate 82Q and the mutant 82Q/15b, tolerance to toxicants may be inducible, since changes in cellular components were detected during growth in

the absence and presence of toxicants.

Isolate 82Q showed a relatively low resistance to copper. During growth in the presence of low concentrations of copper, distinct changes were observed in the extracellular polysaccharide produced, particularly a marked reduction in the uronic acid content and changes in the interactions with ions. This might possibly result in a decrease in the non-specific uptake of copper ions and may affect attachment properties.

Alterations in major outer membrane proteins and also some minor proteins occurred during growth in the presence of copper. This may indicate that control of uptake mechanisms was an attempt to confer tolerant properties on cells of this isolate. However, since 82Q did not tolerate higher levels, this does not appear to be an effective mechanism.

The mutant 82Q/15b did tolerate higher levels of copper ions. The extracellular polysaccharide produced by this strain under routine growth conditions differed from that of the parental strain, showing a reduction in uronic acid content and changes in the ionic interactions. In addition, changes in the major outer membrane proteins and in some minor proteins indicate that, again, the strategy for tolerance may involve control of the uptake of copper by the cell. However, since the mutant can tolerate up to 1.0mM copper sulphate, it appears to have a more successful mechanism. The species of protein involved may be responsible for this since the basic strategy seems to be similar.

Isolate S3/A51 also showed a relatively high level of resistance to copper. The extracellular polysaccharide produced by

this isolate differed from that of isolate 82Q in several ways. It contained a higher ratio of fucose residues, differences were seen in the acyl components, and there was less tendency for ionic interactions to occur. If the strategy for tolerance involved this polymer, the possible structural and chemical properties it possesses may be responsible in some way for preventing the uptake of copper by the cell. This might involve the chelation of the metal ions by the polymer, or perhaps the polymer acts as a physical penetration barrier to the metal ions, since few ionic interactions were observed.

This isolate also had the ability to produce an altered polysaccharide in the presence of copper ions. Slight increases were observed in the acetate and uronic acid content, but the most notable change was an increase in the ratio of galactose residues. This may lead to structural changes resulting in further exclusion of copper ions.

Contrary to isolate 82Q, the major outer membrane proteins produced by isolate S3/A51 in the presence of copper ions were of the same molecular weight as those produced under routine growth conditions. However, some changes in minor outer membrane proteins were observed. In particular, a copper-binding protein with a relative molecular weight in the 50K region appeared to be repressed during growth in the presence of copper. Isolate 82B, which also tolerated relatively high concentrations of copper, however showed no changes in the outer membrane proteins produced during growth in the presence of copper. Tolerance to copper in isolate S3/A51 may involve repression of specific transport systems and, again,

prevention of binding and uptake of copper was indicated as a strategy for tolerance.

Less information has been obtained on the possible mechanisms of tolerance to organotin. It has not been determined in these studies whether tolerance properties are stably inherited in the case of organotin. However, a recent report by Anisimova et al. (1985) indicated that resistance to organotin compounds in pseudomonads was determined by plasmids. The ability to produce different cellular components during growth of all isolates examined in the presence of organotin suggested that tolerance properties may be inducible.

At the relatively low concentrations of DMTC to which isolates 82B, 82Q and S3/A51 were subjected, the effect on growth was similar in all three isolates. However, the response of the isolates with respect to the production of extracellular polysaccharide and outer membrane proteins differed. Isolate 82Q responded by producing a polysaccharide with a marked reduction in uronic acid content as compared to that produced in the absence of toxicants. In addition, as in the response to copper ions, changes in the major outer membrane proteins and in some minor proteins occurred. These observations may indicate an attempt to confer tolerance by preventing uptake of DMTC. However, since at higher concentrations isolate 82Q showed lower tolerance to DMTC than the other two isolates, this mechanism was not greatly successful.

In the presence of DMTC isolate S3/A51 also produced a polysaccharide with altered properties, namely changes in the ratios of monosaccharide components and an increase in the uronic acid

content. No differences in the outer membrane protein profile was observed. Thus, tolerance may have resulted from a change in the polysaccharide structure such that access of the toxicant to the cell was reduced.

In contrast, isolate 82B showed changes in some minor outer membrane proteins produced during growth in the presence of DMTC. In particular, several proteins with relatively high molecular weights seemed to be induced, and may in some way have protected the cell from the effects of organotin.

The strategies for tolerance to organotin are unclear, but there was some indication that, as is the case for tolerance to copper, control of the mechanisms for binding and uptake of the compound were involved.

## 4.5 Conclusions

The work presented here gives some indication of the actions of toxicants used in some antifouling paints on marine bacteria. Results suggest that the sites of action of copper ions include the processes of cell wall synthesis and cell division, and biosynthetic pathways for extracellular polysaccharides. The sites of action of organotin remain to be elucidated, though one effect appeared again to be on the biosynthetic pathways for extracellular polysaccharide production.

Tolerant bacteria have been isolated, but toxicants were still able to cause a reduction in attachment of viable cells to surfaces without affecting cell viability, probably due to interacting with

cell surface components involved in the attachment process.

It seems that to develop tolerant properties, marine bacteria attempt to control uptake of the toxicant by various strategies. This method of tolerance would be presumed to be more efficient and economic than mechanisms involving extracellular chelating material. The latter response would require production of large quantities of extracellular material, which would result in a high energy expenditure. Isolate S3/A51 shows the highest levels of tolerance, suggesting that the changes in structure of the extracellular polymer induced in the presence of both toxicants, and the differences in minor outer membrane proteins induced in the presence of copper ions only, result in a successful strategy for tolerance. However it is difficult to ascertain if these products are unequivocally related to tolerance, or if the changes are due to unrelated action of the toxicants. Examination of other resistant species would be useful to determine if similar responses to the toxicants occurred. The isolation of non-resistant mutants of isolate S3/A51 might reveal further information. Studies to determine if such mutants lacked the ability to induce a specific product or products in the presence of toxicants might allow the definite association of such products with tolerance mechanisms.

Immobilized metal affinity chromatography yielded some information on copper-binding properties of outer membrane proteins of isolate S3/A51. This technique may be useful in further studies on metal-binding properties of bacterial proteins. For instance, it would be of interest to determine if the major outer membrane proteins of isolate 82Q, which undergo molecular weight changes

during growth in the presence of toxicants, also show differing copper-binding properties.

Extrapolations of laboratory findings to the natural marine environment are difficult to make. Environmental factors influencing the effect of toxicants on marine bacteria were discussed in Section 1.8.4. Continuous culture techniques could be utilized in order to determine whether microbial behaviour observed under laboratory conditions approximates to that occurring naturally, by manipulation of parameters such as pH, temperature and nutrient concentration. Such techniques have previously been useful in the study of microbial attachment to surfaces (Wardell et al., 1983).

The analysis of cell surface components having a possible role in tolerance to toxicants is by no means complete. Other constituents have been implicated in binding of metals (see Section 1.8.1), and alterations in such components may thus have an influence on toxicity. For example, the fluidity and melting behaviour of the bacterial outer membrane is related to the content and nature of the LPS and its interaction with cations. A mutant of Pseudomonas aeruginosa with an altered LPS has been found to have an increased permeability to some agents (Nikaido & Vaara, 1985). Thus, analysis of LPS and other cell components may lead to further information on mechanisms of tolerance to toxicants in marine bacteria.

The need for an evaluation of marine antifouling paints has previously been suggested (Dempsey, 1981b). The unravelling of the basis of tolerance to the toxicants used in these paints may

eventually lead to their improvement, perhaps by addition of inhibitors of the products which confer tolerant properties.

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